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THE INCIDENCE OF CANCER OF THE LUNG AND LARYNX IN URBAN AND RURAL DISTRICTS.*

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THE background of the present paper is the striking difference (illustrated in Fig. 1) between the trends of mortality in England and Wales due to cancer of the lung and of the larynx. In contrast to the well-known large increase in deaths

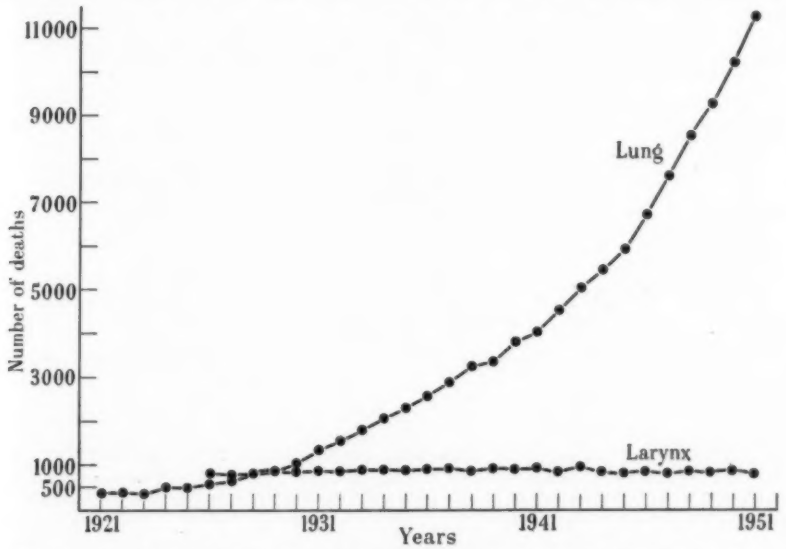

FIG. 1.—Cancer of the lung and of the larynx. Males, England and Wales, 1921–1951.

ascribed to lung cancer, for cancer of the larynx there has been little change over the past twenty years.

The preceding papers in this series (Kennaway and Kennaway, 1951; Kennaway and Waller, 1953) compared the incidence during the period 1946–1949 of

* A preliminary statement of these results was given at a meeting of the European Section of the Union Internationale contre le Cancer at the Institute of Cancer Research, Royal Cancer Hospital, London, on October 19, 1953.

cancer of the lung and larynx as shown by death certificates in four classes of area (London, County Boroughs, Other Urban Districts, Rural Districts) in England and Wales. The Registrar General has now given us material which enables a much more detailed study to be made by comparing the incidence in the three latter classes of area in each of the 11 districts which are set out, with the counties which they comprise, in Table I.

Part I of this paper consists of an analysis of additional material supplied by the Registrar General, which has enabled a more detailed study to be made of the problem. Part II provides some data upon the mortality from cancer of the larynx in recent years and upon the anatomical distribution of this form of cancer in men and in women.

Part I.

Material.

The following analysis consists primarily of a comparison between the mortality during the four years 1946–1949 ascribed to cancer of the lung and larynx in the 11 geographical regions of England and Wales used by the Registrar General up to 1949. These regions are defined as shown in Table I and Fig. 2.

Table I.—*The* 11 *Regions of England and Wales as used by the Registrar General until* 1949.

North I.
Durham.
Northumberland.

North II.
Cumberland.
Westmorland.
Yorkshire E. Riding.
Yorkshire N. Riding.

North III.
Yorkshire W. Riding.

North IV.
Cheshire.
Lancashire.

Midland I.
Gloucestershire.
Herefordshire.
Shropshire.
Staffordshire.
Warwickshire.
Worcestershire.

Midland II.
Derbyshire.
Leicestershire.
Northamptonshire.
Nottinghamshire.

Wales I.
Brecknockshire.
Carmarthenshire.
Glamorganshire.
Monmouthshire.

Wales II.
Anglesey.
Caernarvonshire.
Cardiganshire.
Denbighshire.
Flintshire.
Merionethshire.
Montgomeryshire.
Pembrokeshire.
Radnorshire.

South-East.
Bedfordshire.
Berkshire.
Buckinghamshire.
Essex.
Hertfordshire.
Kent.
London.
Middlesex.
Oxfordshire.
Hampshire.
Surrey.
Sussex.
Wight, Isle of.

East.
Cambridgeshire.
Huntingdonshire.
Lincolnshire.
Norfolk.
Rutlandshire.
Suffolk.

South West.
Cornwall.
Devonshire.
Dorsetshire.
Somersetshire.
Wiltshire.

In addition to this geographical division of the country we have made use of the customary rough and ready grading of local authority areas by the level of

FIG. 2.—England and Wales. The broken lines show the boundaries of the Registrar General's 11 regions (see Table I). The unbroken lines are the boundaries of the larger areas into which these regions have been grouped for the first part of the analysis. (G.L. = Greater London.)

urbanization; each of the above regions is divided into three types of areas as follows :

County Boroughs (C.B.) (most of which are towns with a population of 100,000 or over).
Urban Districts (U.D.) (other towns).
Rural Districts (R.D.) (the rest of the country).

In view of the special importance of London and its environs separate figures have been calculated for that portion of the S.E. region known as Greater

London,* that is the inner County of London and the urban ring of Outer London.

The measure of mortality we have used is the Standard Mortality Ratio (S.M.R.). The S.M.R. for a particular sub-division of the country expresses the mortality in that area as a percentage of that for the whole of England and Wales, allowance being made for any differences in the age-distribution. We shall use separate S.M.R.s for each sex. For example, an S.M.R. of 120 for males in a particular area indicates that mortality is 20 per cent higher than for the males of the whole country. It will be seen therefore that the S.M.R. gives no indication of the *absolute* mortality and therefore does not enable direct comparisons to be made between mortality due to different causes or between males and females.

Statistical significance.

The number of deaths (particularly those due to cancer of the larynx in females) are small and therefore some attention must be paid to the question of sampling error. Formal tests of significance would not be appropriate in a survey of this kind, but in order to indicate those instances where the standard error is large, and consequently where particular caution must be exercised in drawing conclusions S.M.R.s based on less than 400 deaths have been shown in italics and those on less than 100 deaths in brackets.†

Comparison of mortality due to cancer of lung and larynx.

For a first study of the figures it is convenient to use a broader grouping than the 11 regions and these have been combined into five groups: Greater London, North, Midland, Wales and the Rest; this latter group consists of the 3 regions East, S.W. and S.E. (less Greater London). In Table II are shown the S.M.R.s for these and also for the classification by level of urbanization. The number of deaths and crude death rates are also given as an indication of the relative importance of the two causes of death; these measures of mortality will not be used in this analysis.

The most striking feature of Table II is the behaviour of the figures for the female larynx as previously described. Whereas for cancer of the lung the male and female figures show similar trends the corresponding figures for the larynx show no such similarity. Thus of the five geographical divisions it is Wales that has the lowest S.M.R. not only for the male larynx but also for the lung (male and female), while for the female larynx the S.M.R. is 202, that is, twice the average for the whole country. This tendency is even more remarkable in the urbanization analysis; here there is a noticeable similarity between the three sets of figures for the male and female lung and the male larynx, which show a positive association between urbanization and mortality; for the female larynx the exact contrary is true. It may be remarked that Stocks (1939) records even greater

* Greater London (as defined, for instance, in the 1951 Census Reports) consists of the Administrative County of London, the County of Middlesex and parts of Essex, Herts, Surrey and Kent.

† An S.M.R. based on 400 deaths can be taken as having a coefficient of variation of 5 per cent. If such an S.M.R. is 120 it may be considered that the true value will probably lie within the range 120 ± 12. For 100 deaths the coefficient of variation is 10 per cent and for 25 deaths is 20 per cent. (See Registrar General's *Statistical Review of England and Wales*, 1940–1945, Text, Medical, p. 23.)

TABLE II.—*Mortality in England and Wales* (1946–1949) *due to Cancer of the Lung and Larynx.*

	Cancer of lung.		Cancer of larynx.	
	Male.	Female.	Male.	Female.
Total Registered Deaths, England and Wales	32,547	7,097	3,296	1,115
Average death-rate (per million)	409	80	41	13
Standard Mortality Ratio (S.M.R.):				
England and Wales	100	100	100	100
Greater London	137	132	113	*72*
North	100	98	100	120
Midland	93	93	103	*84*
Wales	79	*69*	*83*	*202*
Rest	83	89	94	*88*
County Boroughs (Outside Greater London)	117	108	118	*106*
Urban Districts (Outside Greater London)	86	88	93	*96*
Rural Districts (Outside Greater London)	62	74	76	*128*

Notes.—(i) For definitions of regions see text. (ii) S.M.R.s shown in italics are based on 100–400 deaths and are therefore subject to a coefficient of variation of 5–10 per cent. No S.M.R. in this table is based on fewer than 100 deaths.

differences for males, between urban and rural populations. The following are the Equivalent Average Death-Rates (per million) for men due to cancer of the lung, in the ten years 1921–1930, calculated from Stocks's figures:

County of London	140
County Boroughs	86
Urban Districts	67
Rural Districts	43

Stocks does not, however, give any rates for females.

Some recent figures given by McKinlay (1953, personal communication) for mortality in Scotland show that of nine major sites relevant to men and women the larynx is the only one with a lower mortality among women in the town than the country. Table III shows the urban standardized mortality calculated as a proportion of the rural mortality for these nine sites.

TABLE III.—*Ratios of Urban to Rural Mortality in Scotland* (1950–1952) *due to Cancer of Nine Major Sites affecting Men and Women.* (McKinlay, 1953, personal communication.)

	Urban/Rural mortality ratio.	
	Male.	Female.
Site:		
Buccal cavity and pharynx	1·43	1·08
Oesophagus	1·47	1·27
Stomach	1·30	1·08
Intestine	1·23	1·11
Rectum	1·20	1·09
Larynx	1·96	0·71
Lung	2·17	1·93
Skin	0·88	1·17
Bone and connective tissue	1·30	1·15

Notes.—(i) The ratios are calculated from the appropriate numbers of deaths, standardized by age and sex. (ii) Urban rates are calculated from the four cities of Glasgow, Edinburgh, Dundee and Aberdeen, and rural rates from the counties less the cities and large burghs.

For comparison with the figures given in this paper for England and Wales, the C.M.R.s for Scotland* corresponding to the ratios for mortality due to cancer of the *lung* are as follows :

	Urban.	Rural.
Male	144	66
Female	141	73

The number of deaths due to cancer of the larynx is not sufficiently great to justify the calculation of C.M.R.s for this site.

In spite of the small number of deaths due to cancer of the female larynx there is no doubt, then, of the reality of these differences between mortality in different parts of the country due to cancer of the lung and larynx. The next step is to see whether the geographical or the urbanization factor is the more important ; this can be done by a study of Table IV and Fig. 3, which give separate S.M.R.s for each of the five grouped regions, divided into C.B.s, U.D.s and R.D.s.

TABLE IV.—*Standard Mortality Ratios, England and Wales* (1946–1949) *due to Cancer of the Lung and Larynx.*

		Cancer of lung.		Cancer of larynx.	
		Male.	Female.	Male.	Female.
Greater London :	Administrative County	160	150	*147*	*(56)*
	C.B.	136	*113*	*(97)*	*83*
	U.D.	119	121	*(87)*	
North :	C.B.	124	114	120	*120*
	U.D.	83	88	*87*	*110*
	R.D.	57	*61*	*(62)*	*(149)*
Midland :	C.B.	115	109	*117*	*(87)*
	U.D.	86	*87*	*97*	*(66)*
	R.D.	61	*71*	*84*	*(105)*
Wales :	C.B.	120	*(82)*	*(103)*	*185*
	U.D.	74	*68*	*(78)*	
	R.D.	*60*	*(60)*	(77)	*(242)*
Rest :	C.B.	100	*93*	*112*	*(83)*
	U.D.	91	93	*100*	*75*
	R.D.	65	83	*77*	*109*

Note.—S.M.R.s shown in italics are based on fewer than 400 deaths and are therefore subject to a coefficient of variation of more than 5 per cent. Those also shown in brackets are based on 25–100 deaths and have a coefficient of variation of 10–20 per cent. No S.M.R. in this table is based on fewer than 25 deaths.

It is clear from Fig. 3 and Table IV that urbanization is the predominating factor : in each region, the mortality due to lung cancer increases from the rural to the urban areas and although the same is true of the male larynx, when we look at the figures for the female larynx we find the exact contrary ; in every case the highest mortality is in the rural districts. The comparison is particularly striking when we look at the S.M.R.s for the County of London (the most densely populated of all our areas) and then at those for the sparsely populated Rural Districts in Wales and the North.

* For the purposes of this comparison the differences between the C.M.R. (Comparative Mortality Ratio) and the S.M.R. may be ignored.

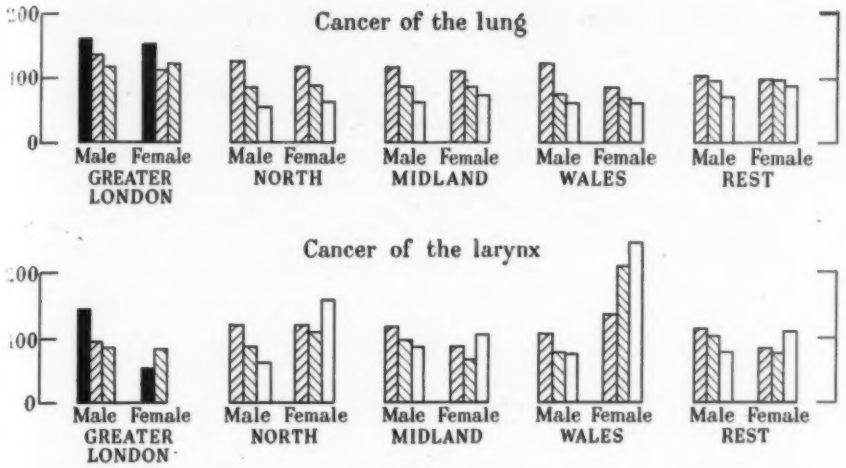

FIG. 3.—Cancer of lung and larynx. England and Wales. Standard Mortality Ratios, 1946–1949.
(England and Wales = 100 : for each disease and sex.)

London Administrative County. Urban Districts.

Rural Districts. County Boroughs.

We have said that urbanization is the predominating factor, implying that it is more strongly associated with the mortality than is the geographical location. But here we have a difficulty, for our classification only allows for gross differences in the level of urbanization between one region and another and we cannot necessarily ascribe any residual differences to purely geographical factors. For example it would seem likely that some at least of the difference between the lung S.M.R.s in the C.B.s of the Midland Region and those of the Rest are due to the fact that in the latter there are no large conurbations, and many of the C.B.s are in fact medium-sized market towns. We cannot say whether the extremely high figures for the female larynx in Wales (where the population at risk is in any case small) are more than an extreme manifestation of the reverse urbanization gradient or whether they might be due, for instance, to some ethnographical factor.

Further study of cancer of the lung.

Another feature that emerges from the diagram is, that for cancer of the lung the urbanization gradient is consistently steeper in the men than in the women. In order to explore this point more fully we can make use of the greater numbers of deaths for the lung and take the analysis one step further. In Table V the S.M.R.s are given for each of the sub-divisions of the 11 regions and with Greater London divided into the County of London and the ring of Outer London. In every region but one the male S.M.R. is greater than the corresponding female

S.M.R. in the C.B.s, and in all but two it is smaller than the female S.M.R. in the R.D.s.

TABLE V.—*Standard Mortality Ratios, England and Wales* (1946–1949), *due to Cancer of the Lung.*

	C.B.		U.D.		R.D.	
	Male.	Female.	Male.	Female.	Male.	Female.
London Administrative County	160	150	—	—	—	—
Outer London	136	*113*	119	121	—	—
North I	101	*107*	75	*74*	*55*	(*61*)
II	123	(*102*)	*72*	(*97*)	*45*	(*84*)
III	121	*113*	77	*79*	*60*	(*84*)
IV	132	118	92	*95*	*69*	(*22*)*
Midland I	118	*112*	90	*85*	60	*67*
II	105	*97*	80	*90*	*63*	(*77*)
Wales I	120	(*82*)	69	(*63*)	*65*	(*61*)
II	—	—	*87*	(*78*)	*55*	(*60*)
South-East (less Greater London)	107	*103*	97	99	76	*96*
East	*95*	(*76*)	85	(*88*)	*50*	(*65*)
South-West	*78*	(*73*)	74	*80*	58	*72*

Notes.—(i) See note to Table IV. The only S.M.R. in this table based on fewer than 25 deaths is that marked with an asterisk (*) which is based on only 17 deaths. (ii) There are no C.B.s in Wales II. (iii) For the sake of convenience the S.M.R.s for London appear in the column headed "C.B."

The difficulty of making comparisons between the regions has already been discussed: many of the differences between regions would almost certainly be lessened if we had a more sensitive measure of urbanization. Such a measure is suggested by Stocks (1952) who has used the *size* of conurbations, which he has shown (with mortality figures for these same four years) is highly correlated with the prevalence of cancer of the male lung. His method was to group together all C.B.s with a common boundary, such as Manchester, Salford and Stockport, and treat such blocks as single units. For this particular conurbation the C.M.R.* is 159, compared with the S.M.R. of 160 for the County of London.

It would be interesting to make a similar analysis with the Urban Districts (or better with the County Boroughs and Urban Districts combined) but the labour would be prohibitive. It may be mentioned in this connection that since 1950 the Registrar General has adopted a more realistic classification, which will considerably simplify the study of many problems. Under the new method the towns of England and Wales are divided, without regard to administrative status, into six "conurbation areas", and "urban areas" of three different sizes.

When we come to the Rural Districts another method suggests itself. Here we are dealing with a more nearly homogeneous distribution of people over the countryside, and we can justifiably compare the mortality with the density of population as measured in terms of numbers of persons per acre.†

In Table VI the figures are compared with the male and female S.M.R.s due to cancer of the lung in the Rural Districts of the 11 regions, which are arranged in descending order of population-density. In the males there is a high correlation ($r = 80$) and this is illustrated in Fig. 4. In North IV and Midland II, where

* See footnote to p. 186.

† A similar comparison can, of course, be made for the C.B.s and U.D.s but this would not be expected to yield any information as the average population per acre of towns and cities depends more on how the boundaries happen to have been drawn than on the true density.

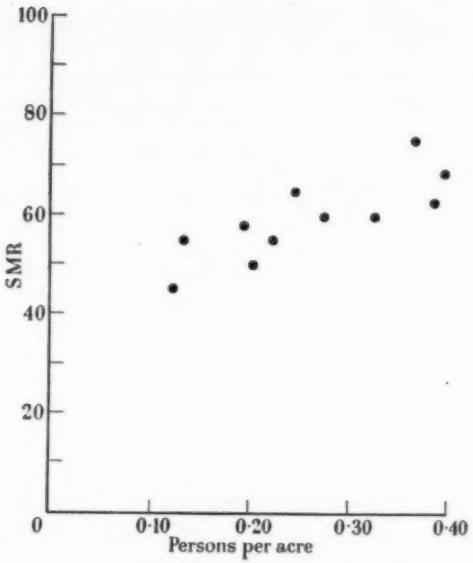

FIG. 4.—Cancer of the lung in males. Rural Districts of England and Wales. Relation of S.M.R. to Population Density.

much of the "countryside" is industrialized, and in the S.E. with its large dormitory areas, the male S.M.R.s for cancer of the lung are all higher than in the sparsely populated S.W., Wales II and North II. In females the absence of such a clear association is partly a reflexion of the lesser gradient already noticed for women and partly due to the larger sampling errors to which these S.M.R.s are subject; on the other hand the remarkably low female S.M.R. for North IV certainly seems anomalous, even when account is taken of the small experience on which it is based.

TABLE VI.—*Standard Mortality Ratios, England and Wales* (1946–1949), *due to Cancer of the Lung in the Rural Districts of the* 11 *Regions compared with the Average Population Per Acre.*

Region.	N. IV.	M. II.	S.E.	N. III.	M. I.	W. I.	N. I.	E.	S.W.	W. II.	N. II.
Persons/acre	·39	·38	·36	·32	·27	·24	·22	·20	·19	·13	·12
S.M.R.—											
Male .	*69*	*63*	76	*60*	60	*65*	*55*	*50*	58	*55*	*45*
Female .	*(22)*	*(77)*	*96*	*(84)*	*67*	*61*	*(61)*	*(65)*	*72*	*(60)*	*(84)*

Note.—The figures for the density of population were calculated from the National Register of September 29, 1939 (H.M.S.O., 1940). This was used in preference to the 1951 Census owing to the extensive boundary changes which came into force in 1950. The comparability of the 1939 population figures with the mortality figures for 1946–9 will, of course, be affected by the war-time movements of population, but these were not very great in the rural districts.

DISCUSSION.

Two main points have emerged from this analysis: first that many of the differences that can be detected in the mortality due to cancer of the lung and

larynx between one part of the country and another are due to differences in the level of urbanization. Whether this applies to all the differences we cannot yet say; in particular there is a suggestion that other factors may be at work in Wales. The second point is that the exact form that this association takes is not the same in men as in women. For cancer of the female lung the urbanization gradient is less steep than in males, though both are positive; for the larynx the slope is positive in males and negative in females. These facts are clearly relevant in the search for the cause or causes of these forms of cancer, whether this be smoking, atmospheric pollution, or some occupational or social factor. If for instance we assume that one factor, say smoking, is responsible for the differences between mortality in town and country then it must be shown, not only that the urbanization gradient of smoking is almost the same in all parts of the country (see Fig. 3) but also that the gradient is greater in men than in women. A possible explanation is that there are two aetiological factors at work, the one positively correlated with urbanization (A) and the other negatively (B). The relative importance of these factors would then be as follows:

Cancer of the lung—	Male:	A predominating.
	Female:	A predominating but to a less extent than in males.
Cancer of the larynx—	Male:	A predominating.
	Female:	B predominating.

This is of course an over-simplification and there may be no factor affecting both sites; we must in any case postulate a third factor (C) giving rise to the increasing mortality due to cancer of the lung but not affecting the larynx. It might be that the interaction between the three factors accounted for the different rates of increase in men and women.

The only one of the likely factors on which the Registrar General's figures can throw much light is that of occupation and social class. In the 1931 Decennial Supplement the Registrar General wrote:

"Other sites which manifest an unmistakable social class gradient amongst males, tending to maximal levels amongst the unskilled classes, are the larynx, skin, scrotum and penis, which are sites exposed to external irritants. The mortality ratio for the larynx ranged from 60 for males of Class I to 143 for males of Class V, but it is significant that married women showed no significant social class gradient, thus suggesting that the factors responsible for the excess of cancer of this site amongst unskilled males are directly associated with occupation."

On the other hand there was in 1931 no marked social gradient for cancer of the lung.

What connection is there between the occupational factor and urbanization? We cannot answer that question in the absence of mortality rates analysed by urbanization simultaneously with occupation. The proportions of the social classes in the town and country are not the same, as may be seen from the following figures for males in 1931:

	Percentages.			
Social classes	I and II.	III.	IV and V.	Total.
County Boroughs (and Greater London)	15	52	33	100
Urban Districts	15	51	34	100
Rural Districts	21	38	41	100

It might well be that the increased mortality of men in the towns is associated with the greater proportion of those classified in Grade III, but we shall have to

wait for a fuller elucidation of the problem until all the results of the 1951 Census are available. It is to be hoped that the Registrar General's next Decennial Supplement, which will be based on these results, can provide some of the answers.

Part II.

Cancer of the Larynx.

Comparison of the recorded liability of men and women to "cancer of the larynx" does not provide a simple contrast of the occurrence of the same disease in the two sexes. Cancer of the larynx in men, and in women, are to a large extent different diseases, the latter being more prone to occur in the extrinsic, and especially in the post-cricoid, area. The data given in an earlier paper (Kennaway and Kennaway, 1951) can now be amplified.

1. During the last 25 years cancer of the larynx, in both sexes, has been unaffected by the factor or factors which have produced a great increase in deaths attributed to cancer of the lung (Fig. 1 and 5). Thus the number of deaths from cancer of the larynx has shown no great change in either sex between 1933 and 1948 (Table VII). In view of the ageing population, this fairly constant level indicates a fall in mortality which has become evident, in both sexes, in the last 3 years (1950–1952) for which figures are available; possibly this change is due in part to more successful treatment.

Table VII.—*Deaths from Cancer of the Larynx, England and Wales*, 1933–1952.

	Males.	Means.	Females.	Means.
1933	882		236	
1934	902		243	
1935	888		232	
1936	907		285	
1937	922		240	
1938	910	900	268	259
1939	962		293	
1940	888		267	
1941	906		269	
1942	838		255	
1943	852		281	
1944	819		280	
1945	823		279	
1946	853	828	258	280
1947	790		286	
1948	851		288	
1949	813		284	
1950	809		182	
1951	805	789	202	191
1952	752		189	
Mean of all	859		256	

Note.—The lower level of totals from 1940 onwards is due in part to the change in the procedure used for selecting the cause of death when two or more are mentioned on the certificate.

2. Cancer of the lung, and of the larynx in males show similar relations to urban and rural conditions (Table III and Fig. 3). In the case of cancer of the lung the greater incidence in towns has been attributed to greater consumption of

tobacco ; but the constancy of the mortality from cancer of the larynx in recent years suggests that smoking has no carcinogenic effect upon the larynx and hence some other reason must be sought for the difference between town and country.

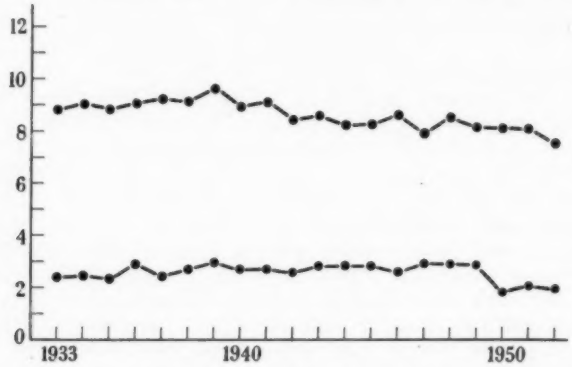

FIG. 5.—Deaths from cancer of larynx in hundreds. England and Wales, 1933–1952.

3. The anatomical and sexual distribution of cancer of the larynx is illustrated by further unpublished material for which we are indebted to Dr. R. B. Terry of this hospital, and to Mr. V. E. Negus, who has given us data from King's College Hospital and from his private practice (Table VIII and Fig. 6). All our material

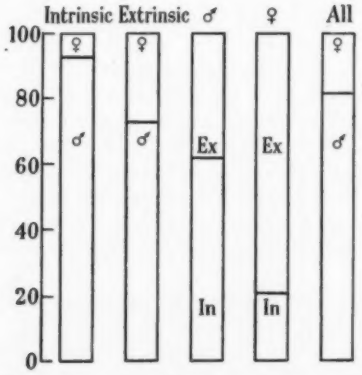

FIG. 6.—Anatomical distribution of cancer of the larynx in men and women based on 964 cases (Semon, Terry, Negus, Thomson and Colledge).

(964 cases) was obtained in London ; figures from other areas would be very desirable. Unfortunately death certificates do not provide a basis for the separation of extrinsic and intrinsic cancers ; one must therefore rely upon the records of individual hospitals and surgeons, and such sources are notoriously liable to disturbing factors.

TABLE VIII.—*Anatomical Distribution of Cancer of the Larynx in Men and Women.*

964 Cases of Cancer of the Larynx.

	Intrinsic.	Per cent.	Extrinsic.	Per cent.	Total.	Per cent.	Intrinsic per cent of total.
Semon, 1878–1906:							
Male	124	91·2	53	70	177	83·5	70
Female	12	8·8	23*	30	35	16·5	34·3
Total	136	100·0	76	100·0	212	100·0	
St. Bartholomew's Hosp., 1935–1953:							
Male	131	90·3	110†	73·3	241	81·7	54·4
Female	14	9·7	40	26·7	54	18·3	25·9
Total	145	100·0	150	100·0	295	100·0	
V.E. Negus, 1925–1951:							
Male	174	95·2	104	61·2	278	79	62·6
Female	8	4·8	66	38·8	74	21	10·8
Total	182	100·0	170	100·0	352	100·0	
Thomson and Colledge, 1930:							
Male	94	89·5					
Female	11	10·5					
Total	105	100·0					
Totals (Thomson and Colledge omitted):							
Male	429	92·7	267	67·2	696	81	61·7
Female	34	7·3	129	32·8	163	19	20·9
Total	463	100·0	396	100·0	859	100·0	

* 18 of these postcricoid.

† 1950–53:

	Male.	Female.
Postcricoid	2	8
Other extrinsic	18	1

4. If the figures in Table VIII are representative, they may be said to show that—

(*a*) cancer of the larynx is four times as common in men as in women;
(*b*) in men, intrinsic cancers are nearly twice as common as extrinsic, whereas in women extrinsic are four times as common as intrinsic;
(*c*) as a consequence of (*a*) and (*b*), intrinsic cancers appear nearly thirteen times as often in men as in women* and the extrinsic twice as often in men as in women.

The conventional inclusion of cancers of the retro-cricoid region among laryngeal tumours is very unfortunate. "The term (cancer of the larynx) should be restricted to the so-called 'intrinsic' group of cancers. The extrinsic group are of pharyngeal origin, require separate and distinct classification, and should be excluded from any discussion on laryngeal cancer" (Lederman, 1950).

The Paterson-Kelly syndrome.

Any comparison of cancer of the larynx in men and in women requires some consideration of the syndrome, confined to women, of dysphagia, due to "organic stenosis at the level of the cricopharyngeal sphincter or immediately beyond it" (Bingham and Logan, 1953), atrophy and ulceration of the mucous membranes of the

* Of 178 cases of intrinsic laryngeal cancer treated by teleradium at the Radiumhemmet, Stockholm, during 1935–45, 88·2 per cent were in men (Jacobsson, 1952).

mouth, tongue, and pharynx, angular stomatitis, hypochromic anaemia, achlorhydria, splenomegaly, and spoon nails, in which carcinoma of the post-cricoid region may develop in later years. This condition was first described independently by Paterson from Cardiff (1919) and by Brown Kelly from the Western Infirmary, Glasgow (1919) and has become known by the names of two later investigators as the "Plummer-Vinson" syndrome. Neither Paterson (1919, 1937) nor Brown Kelly (1919, 1931) suggest, either in their earlier or later papers, that this affection is especially common in their own districts, though any such frequency would have assisted recognition. This syndrome is of quite especial interest in cancer research because the features present during the earlier years may indicate a precancerous metabolic state, which may be treated successfully by improved feeding and administration of iron (See for instance Hallén, 1938; Waldenstrom, 1938, and Elkeles, 1942). The following section represents an attempt to collect the literature on the Paterson-Kelly syndrome in relation to cancer; no attempt has been made to introduce any uniformity into the anatomical classification adopted by the various authors.

Ahlbom (1937) records, from the Radiumhemmet, Stockholm (1931–1936), the incidence of carcinoma of the post-cricoid region. (*a*) Cancers of the hypopharynx are more frequent in women, in whom also a much higher proportion (90 per cent) of these tumours affect the post-cricoid area, than in men (10 per cent) (Table IX). (*b*) Seventy-five per cent of cancers of the larynx and oesophagus in women are accompanied by other signs of the syndrome (Table X).

In a later paper Ahlbom (1941) defines hypopharyngeal cancers as those arising between the vallecula and the upper end of the oesophagus, excepting the

TABLE IX.—*Carcinoma of Meso- and Hypopharynx, Larynx and Oesophagus. Cases at the Radiumhemmet*, 1931–1936 (Ahlbom, 1937).

Carcinoma.	Male.	Female.	
Tonsil	16	7	
Other mesopharynx	0	8	About 10 per cent of male and 90 per cent of female were post-cricoid.
Hypopharynx	50	74	
Larynx	47	8	
Oesophagus	24	16	
	137	113	

TABLE X.—*Female Patients at the Radiumhemmet*, 1931–1936 (*Ahlbom*, 1937).

Carcinoma.	Total cases.	Not examined for anaemia.	Examined.	Plummer-Vinson syndrome.	Simple achylic anaemia.*	Negative.
Tonsil	7	2	5	3	0	2
Other naso-pharynx	8	3	5	5	0	0
Hypopharynx	74	25	49	37	5	7
Larynx	8	0	8	1?	1?	6
Oesophagus	16	10	6	3	0	3
	113	40	73	49	6	18
				55=75 per cent of those examined.		

* One must infer from the totals that these cases showed no other signs of the syndrome.

intrinsic laryngeal carcinomas. His series of 235 cancers of this region was derived from 129 women and 106 men seen at the Radiumhemmet during 1937–39, (Table XI). Sideropenia (this term includes the Plummer-Vinson syndrome and simple achlorhydric anaemia) . . . " in this country is to be regarded as the cancer-predisposing factor of decisive significance in these female patients. In practically

TABLE XI.—*Cancers of Hypopharynx, Radiumhemmet*, 1931–1947 (*Ahlbom*, 1941 *and Jacobsson* 1951).

				Cancers of hypopharynx.		
	Male	Female per cent of female	Female per cent of total.	Upper.	Lower (post-cricoid).	Sideropenia.
Ahlbom, 1931–1936	50	74	60		20 per cent of male 90 per cent of female	90 per cent of post-cricoid.
„ 1931–1939	106	129	55		"Practically all" hypopharyngeal cancers in sideropenic female	
Jacobsson, 1939–1947	119	203	63	85 per cent male	20 per cent female	10 per cent of male. 90 per cent of female.

all of these women the site of the cancer is what is referred to as post-cricoid. This form is extremely rare in the male patients where the cancer develops in the sinus piriformis, the aryepiglottic folds, on the posterior arytenoid regions, as well as on and around the epiglottis." These two papers by Ahlbom (1937, 1941) contain valuable summaries of the literature.

Jacobsson (1951) has given more data from the Radiumhemmet (Table XI). He says: " The border line between the upper, and lower, hypopharynx, . . . " (as defined above by Ahlbom) " has been drawn at the superior border of the cricoid cartilage." The material consists of 322 cases (119 in males and 203, or 63 per cent, in females); about three-quarters of the tumours were in the lower hypopharynx, but to define the exact site of origin may not be possible. 85 per cent of the cases in the upper, and only 20 per cent of cases in the lower, hypopharynx were in males. " In our cases 90 per cent of the women and 10 per cent of the men showed definite signs and symptoms of sideropenia " (apparently this statement applies to tumours of all parts of the hypopharynx).

Jacobsson gives some figures from other countries. In Ireland, Norway, Scotland and Sweden, women provide 40 to 60 per cent of cancers of the hypopharynx, whereas in France and Switzerland this conditions in women is very rare. The range given is so wide (from 1 per cent in France to 60 per cent in Sweden) that one would like to be sure that the diagnostic criteria are the same. Hallén (1938) speaks of "the widespread achlorhydria in the northern provinces of Sweden."

Simpson (1939), whose cases " were all from Hull and the immediate area surrounding in the East Riding of Yorkshire " (personal communication), says: " on examining the records of the cases of this syndrome referred to me for dysphagia and oesophagoscopy, I find that at least 50 per cent either had or developed a carcinoma. Of the 18 cases seen, 10 had either a carcinoma or developed one while under observation. Of the 10 carcinomas, four were in the post-cricoid area, one was in the lower third of the oesophagus, and five were located at the cardiac end of the stomach and oesophagus."

Pilcher (1948) gives the following details of 100 consecutive cases of carcinoma of the pharynx seen at University College Hospital, London :

TABLE XII.—*Carcinoma of Pharynx* (Pilcher, 1948).

		Epilaryngeal.	Post-cricoid.	Total.
Male . .	70	Pyriform fossa, 42 . Lateral wall, 9 .	0 .	70
Female		1 .	29 .	30

Lederman (1953) has discussed the anatomy of the pharyngo-laryngeal groove and sinus pyriformis. "Tumours arising from the third or lowest part of the groove can scarcely be distinguished from other tumours of the post-cricoid space and should be regarded as forming part of this latter group. . . . Tumours of the upper and mid parts of the groove can be conveniently regarded as a single group and the term "sinus pyriformis" cancer applied to this joint group, the "sinus pyriformis" for this purpose being regarded as extending from the upper border of the pharyngo-epiglottic fold above to the crico-pharyngeal fold below." He gives the following data, which show the sexual distribution, in his material, immediately above the post-cricoid area. The sex-ratio (15 : 1) is almost the same as that (13 : 1) in Stanford Cade's (1953) series (Table XIII).

Cancer of Sinus Pyriformis.
112 previously untreated cases.
Royal Cancer Hospital, London. 1933–1951.
Male 105. Female 7.

A full discussion of anatomical relationships has been given in a paper by Lederman (1954).

Stanford Cade (1953) gives the following analysis of 403 cases seen by him in London of squamous-celled carcinoma of the laryngo-pharynx.

TABLE XIII.—*Carcinoma of Laryngo-Pharynx*, 1930–1952 (*Stanford Cade*, 1953).

	Total.	Men.	Women.
Epilaryngeal . .	77 .	70 .	7
Lateral pharyngeal .	47 .	42 .	5
Pyriform fossa . .	167 .	155 .	12
Post-cricoid . .	112 .	30 .	82
Total . .	403 .	297 .	106

Bingham and Logan (1953) in Belfast, met with 16 cases of the syndrome in 5 years, of which 2 developed post-cricoid carcinoma ; they say that the condition is "relatively common in the north of Ireland . . . the social class and the level of intelligence are below average . . . Courses of iron produce no permanent improvement . . . if not repeated twice a year . . . these people have not been able to eat meat for years . . . the lumen at the stricture being sometimes only a few millimetres in diameter."

Diet in relation to the Paterson-Kelly syndrome.

Stocks (1939) has examined dietetic conditions in North Wales. "The statistical association between cancer and the amount of fresh milk and certain

fresh vegetables in dietaries which was found some years ago by comparing the diets of 400 cancer patients with those of 400 control patients of the same ages from the same localities (Stocks and Karn, 1933) may help to provide a key to this. Fresh milk and vegetables seem to be very poorly represented in the diet of the bulk of the population of North Wales."

Visitors to Wales, who make no claim to have investigated the matter upon a statistical basis, may get the impression that white bread-and-butter and tea predominates in the diet of many women. Hallén (1938) speaking of diet in relation to the Paterson-Kelly syndrome says: " . . . the problem may be of great importance for the cure of the widespread achlorhydria in the northern provinces of Sweden. According to recent calculations large fractions of the population live on a diet very poor in iron (men 17·5 mg. iron pro die, women only 11 mg)." On a possible anti-carcinogenic action of milk see Hoch-Ligeti (1946, 1952).

Ahlbom (1937) says: " Im Material des Radiumhemmets ist das Verhältnis zwischen der Anzahl von Männern und Frauen bei den in Rede stehenden Karzinomgruppen* ungefähr 1 : 1 bei den ärmeren Patienten, aber ungefähr 5 : 1 bei den Privatpatienten. Unter den ungefähr 100 Fällen von Plummer-Vinsonschen Syndrom und einfacher Achylie-Anämie mit Karzinom, die wir seit dem Jahre 1931 diagnostizierten, waren nur 4 Privatpatienten, eine bemerkenswert geringe Verhältniszahl. Was in der Diät der ärmeren schwedischen Bevölkerung fehlt, sind vor allem eisenhaltige Vegetabilien, Obst und Fleisch."

Bingham and Logan (1953) say " the incidence of hypochromic anaemia in Northern Ireland is considerable and is to be attributed to the inadequate nutrition common in middle-aged women in Northern Ireland and to uncorrected menorrhagia."

A valuable paper, based upon 300 cases of the Paterson-Kelly syndrome observed at the Radiumhemmet, with numerous references to the literature, has been published by Lindrall (1953).

SUMMARY.

1. The mortality due to cancer of the larynx has shown little change in the last 20 years; this contrasts with the well-known increase in that due to cancer of the lung.

2. Mortality due to cancer of the lung and larynx in the County Boroughs, Urban Districts and Rural Districts of different parts of England and Wales is analysed for the period 1946–1949.

3. The Standard Mortality Ratio (S.M.R.) for cancer of the lung in both sexes, and of the larynx in males, increases with increasing urbanization, that is to say, is greater in the County Boroughs than the Urban Districts, and in the Urban Districts than the Rural Districts. Cancer of the female larynx shows exactly the reverse relationship.

4. These trends apply equally when the figures are analysed according to the separate regions, but there are differences between the regions, which may or may not be due to differences in degrees of urbanization undetected by the classification we have used. In particular the S.M.R.s for Wales are very low for the lung and male larynx but very high for the female larynx. The urbanization-gradient is less steep for the female lung than for the male lung.

* Lip, mouth, pharynx, larynx, oesophagus.

5. The mortality due to cancer of the male lung is positively correlated with the numbers of persons per acre in the Rural Districts of the 11 regions of England and Wales.

6. The significance of these findings is discussed in the light of the possible aetiology of cancer of the lung and larynx.

7. The anatomical and sexual distribution of 964 cases of cancer of the larynx is tabulated. Cancer of the larynx in men, and in women, are to a large extent different diseases, the so-called extrinsic group being more frequent in women.

8. The literature upon the relation of the Paterson-Kelly syndrome to cancer, and upon possible dietetic and social factors in this condition, is summarized.

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THE SIGNIFICANCE OF HISTOLOGICAL TYPING IN THE STUDY OF THE EPIDEMIOLOGY OF PRIMARY EPITHELIAL LUNG TUMOURS: A STUDY OF 466 CASES.

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KREYBERG (1952) in a previous paper analysed a series of one hundred consecutive primary epithelial lung tumours of clinical, mainly surgical origin. The tumours were histologically typed and a brief description was given of the diagnostic criteria used. Those tumours were collected during the years 1948 to 1951.

In the present paper this first series will be designated Series I, and a new material of the same origin, Series II, consisting of 133 cases will be added and the total material used in an analysis of the relationship between histological type and epidemiological aspects. Series I has been reduced to 99 cases as one tumour, originally accepted as a primary, was later shown to be a secondary from a breast carcinoma. Further, because of greater experience, one tumour in this series, designated "malignant adenoma," was revised to a small-cell carcinoma, and another changed from the adenocarcinoma to the salivary gland tumour group. The material in Series II has been collected during the years 1951 to 1953. The largest part has been obtained from the Rikshospital, which receives patients from the whole country. Approximately one-sixth of the material has been placed at my disposal by Professor C. Semb at the Surgical Department III and by Prosector E. Hval at the Pathological Laboratory at Oslo City Hospitals, for which I owe my sincere thanks. A few cases only come from other hospitals.

In addition to the two clinical, two post-mortem series have been used in this analysis, namely, the material of Jacobsen (1953) from Oslo City Hospitals, covering the years 1937 to 1946, and the material of Christiansen (1953) from the Rikshospital, covering the years 1925 to 1952/1. All the tumours of these post-mortem materials have been typed by Kreyberg with no knowledge of the clinical data. The present analysis accordingly is based upon 466 cases.

The total figures of each series are comparatively small, but a few features seem to be significant, and possibly important.

The main differences between the clinical and the post-mortem series are (1) the relatively much higher number of adenocarcinomas, 30 per cent of all cases in the latter against less than 10 per cent in the former, and (2) the lower number of squamous cell carcinomas in the post mortem series. The other histological types are remarkably uniform in their occurrence. A study of the two papers dealing with the post-mortem series reveals a greater number of old persons in these materials, as compared to the clinical group. The significance of these differences will be discussed later.

In Kreyberg's (1952) first paper a comparison was made between the occurrence of the different histological types in the Norwegian material and a similar recent material from the Mayo Clinic. The considerable differences were explained

TABLE I.—*Histological Types of Lung Tumours.*

Investigator.	Squamous cell carcinoma.		Large cell carcinoma.		Small cell carcinoma.		Adeno-carcinoma.		Bronchiolar cell carcinoma.		Adenoma salivary gland tumour.		Mixed forms, uncertain.	
	Number.	%	Number.	%	Number.	%	Number.	%	Number.	%	Number.	%	Number.	%
Kreyberg (1952) Series I (99 cases).	44	44·4	8	8·1	18	18·2	6	6·1	3	3·0	16	16·2	4	4·0
Kreyberg Series II (133 cases)	62	46·6	6	4·5	32	24·0	9	6·8	5	3·8	18	13·5	1	0·8
Jakobsen, A. (1937–46) (100 cases) Oslo City Hospital.	19	19	11	11	24	24	30	30	2	2	14	14	—	—
Christiansen T. (1925–52) (134 cases) The Rikshospital	39	29·1	9	6·7	32	23·9	39	29·1	5	3·7	10	7·5	—	—
Foot (1952) Series I, Table IX (123 cases)	55	45	15	12	28	23	14	11	5	4	1	1	5	4
Foot Seriet II, Table X (206 cases).	115	56	18	9	49	23	20	10	1	0·5	0	—	3	1·5
Foot Series IV, Table XII (330 cases)	178	54·0	65	20	41	13	37	11	5	1·5	2	0·7	2	0·7

TABLE II.—*Distribution of Cases of Primary Epithelial Lung Tumours according to Age, Sex and Histological Type of Tumour in 232 Clinical Cases.*

	10–19		20–29		30–39		40–49		50–59		60–69		70–79		80–89		All ages	
	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.	M.	F.
Squamous cell carcinoma	—	—	—	—	2	—	17	—	50	2	28	—	7	—	—	—	104	2
Large cell carcinoma	—	—	—	—	—	—	3	—	3	2	6	—	—	—	—	—	12	2
Small cell carcinoma	—	—	—	—	2	—	8	—	26	2	9	2	1	—	—	—	46	4
Adenocarcinomas	—	—	—	—	—	—	1	2	3	2	2	3	—	2	—	—	6	9
Bronchiolar cell carcinoma	—	—	2	—	—	—	—	—	2	2	1	—	—	1	—	—	5	3
Adenomas Salivary gland tumours	—	1	4	4	4	4	3	4	5	1	1	3	—	—	—	—	17	17
Uncertain	—	—	—	—	—	—	1	1	1	1	—	1	—	—	—	—	2	3
All types	—	1	6	4	8	4	33	7	90	12	47	9	8	3	—	—	192	40

TABLE V.—*Number of Cases Occurring in Each Age Group and Each Sex, given in Absolute Figures from the Norwegian Clinical and the Two Post Mortem Materials.*

Quotients (see Table IV and text) have been calculated from the totals of the three materials and for both sexes jointly.
Population background 1950.

Age groups.	Sex.	Populations (in thousands).	Adenocarciomas.					Adenomas and salivary gland tumours.				
			Materials.			All materials.		Materials.			All materials.	
			K. Number.	C. Number.	J. Number.	Males and Females Number.	Quotient.	K. Number.	C. Number.	J. Number.	Males and Females Number.	Quotient.
10–19	M.	215	—	—	—	—	—	—	1	—	2	0·5
	F.	206	—	—	—	—	—	1	—	—		
20–29	M.	255	—	1	—	1	0·2	4	—	—	9	1·8
	F.	245	—	—	—			4	—	1		
30–39	M.	255	—	1	—	2	0·4	4	1	—	9	1·8
	F.	252	—	1	—			4	1	—		
40–49	M.	220	1	3	3	15	3·4	3	2	5	15	3·4
	F.	227	2	3	3			4	1	—		
50–59	M.	175	3	2	1	20	5·5	5	2	—	8	2·2
	F.	186	2	9	3			1	—	—		
60–69	M.	112	2	4	3	19	7·9	1	—	—	9	3·7
	F.	130	3	5	2			3	1	4		
70–79	M.	68	—	2	7	17	11·3	—	1	1	5	3·3
	F.	82	2	2	4			—	1	2		
80–89	M.	22	—	—	—	—	—	—	—	—	—	—
	F.	31	—	—	—							

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mainly as a result of differences in criteria for typing. In the present Table I, three series from Foot (1952) have been included in order to stress this point. The distribution of histological types in this American material is remarkably similar to the Norwegian clinical series, with one exception, the group adenomas and salivary gland tumours. These tumours occur more than ten times as frequently in all the Norwegian series. The cause of the relatively greater number of these tumours may be manifold and will be discussed later. Suffice it to mention at this point that even in America certain investigators have found these tumours rather frequently.

In Table II is presented the distribution of the different histological types as regards age and sex. The material will be referred to in the following discussion.

TABLE III.—*Sex Distribution of the Different Histological Types in the Clinical Material.*

Squamous cell ca.	Group I	104	males	: 2	Females	. 52	: 1	20 : 1
Large cell ca. .		12	„	: 2	„	. 6	: 1	
Small cell ca. .		46	„	: 4	„	. 11·5	: 1	
Adenocarcinomas .	Group II	6	„	: 9	„	. 0·6	: 1	1 : 1
Bronchiolar cell ca. .		5	„	: 3	„	. 1·7	: 1	
Adenomas: sal. gl. t.		17	„	: 17	„	. 1	: 1	
Uncertain		2	„	: 3	„			

In Table III the findings regarding the sex distribution have been summarized. It will be seen that the different histological types in this clinical material form two main groups with markedly different sex ratio. The Group I tumours show a very great preponderance of males, whereas the Group II tumours show a sex ratio of nearly 1:1.

TABLE IV.—*Number of Cases Occurring in Each Age Group and Each Sex, given in Absolute Figures from the Clinical Material.*

Quotient means the relative figure when the cases are seen on the background of the size of the population in 1950.

Age group.	Sex.	Population (in thousands).	Total lung tumours		Squamous cell tumours.		Large cell—small cell.	
			Number.	Quotient.	Number.	Quotient.	Number.	Quotient.
10–19 .	M. .	215	—	—	—	—	—	—
	F. .	206	1	0·5	—	—	—	—
20–29 .	M. .	255	6	2·4	—	—	—	—
	F. .	245	4	1·6	—	—	—	—
30–39 .	M. .	255	8	3·2	2	0·8	2	0·8
	F. .	252	4	1·6	—	—	—	—
40–49 .	M. .	220	33	15·0	17	7·7	11	4·6
	F. .	227	7	3·0	—	—	—	—
50–59 .	M. .	175	90	51·4	50	28·6	29	16·6
	F. .	186	12	6·5	—	—	—	—
60–69 .	M. .	112	47	42·0	28	25·0	15	13·4
	F. .	130	9	6·9	—	—	—	—
70–79 .	M. .	68	8	11·8	7	10·3	1	1·4
	F. .	82	3	3·6	—	—	—	—
80–89 .	M. .	22	—	—	—	—	—	—
	F. .	31	—	—	—	—	—	—

The occurrence of the different tumour types in relation to age has been examined in some detail. In evaluating the age distribution it will be necessary to relate the absolute numbers to the size of the population in the various age groups. In Table IV this relationship is presented for the whole clinical material, males and females separately, for squamous cell carcinomas in males and for large and small cell carcinomas, likewise in males only. The fact that the tumour material has been collected during the years 1948 to 1953, while the population figures refer

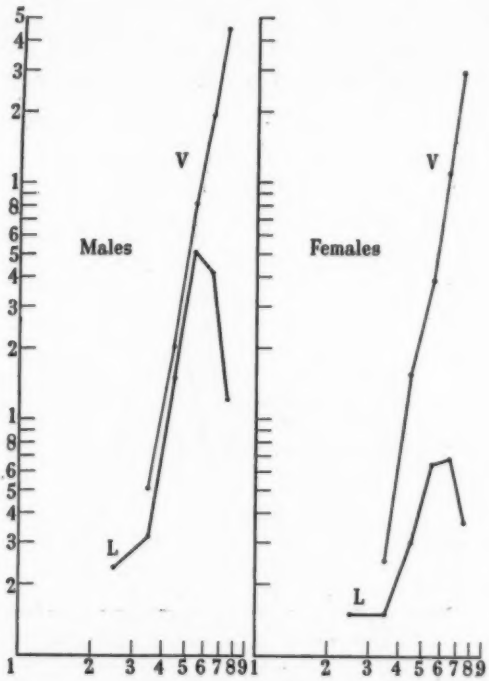

FIG. 1.—Age distribution at the time of diagnosis of all lung tumours (L) and the mortality figures for stomach carcinomas (V) in males and females separately. Double logarithmic presentation.

to the year 1950, is of no importance, as a quotient only is aimed at, permitting to present the relationship and to draw corresponding graphs. The figures do not refer to incidences.

Based upon these quotients, in Fig. 1 curves have been drawn on double logarithmic paper, representing the whole material of the clinical series for males and females separately. For comparison one other curve for each sex has been drawn, namely, the corresponding curves for stomach carcinomas, based upon the mortality figures for Norway, 1950. The reason for this special graphic presentation is the fact, recently stressed in this journal by Nordling (1953) that the total cancer incidence in the different countries plotted in this manner shows a straight line.

This is an expression of the usual occurrence for most human carcinomas, breast carcinoma and uterine carcinomas being the most notable exceptions.

As will be seen, the lung tumour curve in both sexes deviates markedly from the curves drawn for comparison. The distinguishing features are mainly: (i) a gradual increase in the lower age groups, (ii) the straight line rise in the age groups 30–39 to 50–59 is broken by a slight decline in the age groups 60–69, and (iii) followed by a very marked decline in the age group 70–79. In females the irregularity of the lung tumour curve is especially striking.

The first tentative conclusion from this graph is, that the lung tumour curve represents a heterogeneous material, that different biological entities are included.

In Fig. 2 two curves have been plotted in the traditional manner, namely, the curves for the relative age frequency of squamous cell carcinomas and for large cell

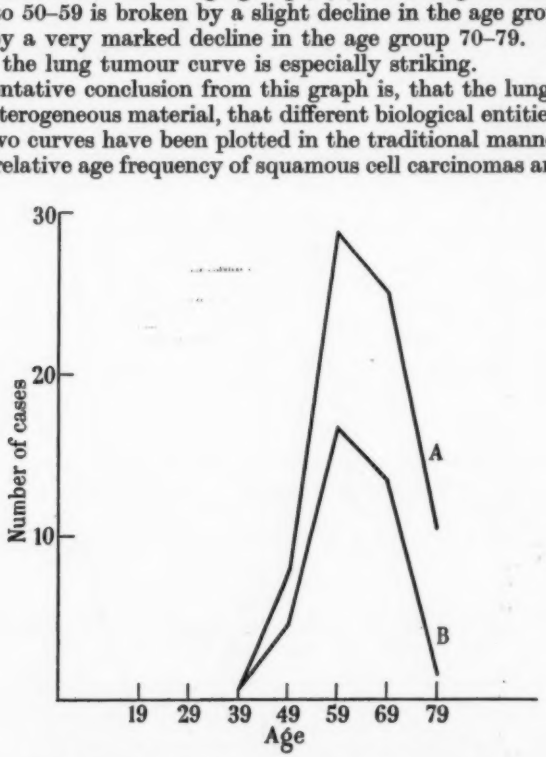

FIG. 2.—Age distribution of squamous cell carcinomas (A), and large-cell and small-cell carcinomas (B) in males only. Clinical material.

and small cell carcinomas jointly, all in males. The figures are given in Table IV The two curves are nearly identical and they indicate that the Group I tumours biologically actually represent a uniform group, in spite of unquestionable histological differences. It will further be seen that these Group I tumours mainly are responsible for the characteristic features of the lung " cancer " curve in general.

The other lung tumour types have been examined in a similar manner. The adenocarcinomas and the sub-group lung adenomas and salivary gland tumours are, however, comparatively few in number in each of all the series, and accordingly subject to considerable chance fluctuations.

In Table V the figures for these tumours in all the three Norwegian materials are presented. The basic figures are given for each material separately. The

quotient has, however, been calculated from the total material, males and females jointly. The few cases which belonged to Christiansen's (1953) as well as Kreyberg's (1952) materials have been included in the latter only. As the sole aim has been to find a picture, as true as possible, of the relative frequency of occurrence of the tumours in the different age groups, such a pooling has been considered permissible. Actually, the pooling probably gives a more accurate picture than the clinical material alone, however great the clinical material might be, because the higher age groups would not be sufficiently represented. This error does not play any role for the Group I tumours, because the number of these tumours in the higher age groups is relatively very small.

In Fig. 3 the quotients have been plotted on the same scale as in Fig. 2, and again the curves show different and characteristic profiles. The adenocarcinomas

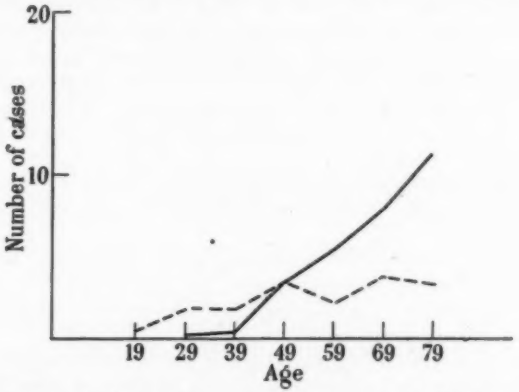

FIG. 3.—Age distribution of adenocarcinomas and adenomas and salivary gland tumours in males and females and in the clinical and post-mortem materials together.

——— = Adenocarcinoma.
- - - - - = Adenomas and salivary gland tumours.

increase in number evenly and steadily with the advancing age. The curve belongs to the same pattern as shown for the stomach carcinomas.

The curve for the subgroup lung adenomas and salivary gland tumours show a strikingly even occurrence in the different age groups, indicating that time is not an important factor in the development of these tumours.

Again we have found that the different histological pictures are accompanied by different biological properties.

We have, further, actually found that the curve for all lung tumours represent a heterogeneous material, that lung " cancer " embraces different biological entities. In the general lung "cancer" curve the adenomas and the salivary gland tumours account for the early and slow rise, and the same tumours, and still more the adenocarcinomas, are responsible for the more gradual decline as seen in the higher age groups.

DISCUSSION.

Squamous cell carcinomas and large and small cell carcinomas, generally regarded as the types of lung tumours connected with special irritants, occur with

a very marked prepondernance in males, and they show the same peculiar age curve. They very rarely occur before late in the third decade and they show to-day a definite decline after the sixth decade, in striking contrast to most human carcinomas. In spite of different histological pictures, admittedly with transitional forms, it is therefore rational to regard these tumours as a biological entity.

Korteweg (1951) in a penetrating analysis of the lung tumour curve in general, which in his material (from England and Wales) to a great extent was stamped by the Group I tumours, explained the unusual shape of the curve as a result of a new carcinogenic situation, having arisen essentially in this century. Clemmesen, Nielsen and Jensen (1953) further have elaborated this point. The unusual fall in the higher age groups is taken to indicate a new carcinogenic situation in development, and the future stages are forecast by the direction of the curve for the age groups 30–39 and 50–59.

The adenocarcinomas, according to our diagnostic criteria : malignant tumours composed of more or less atypical, secreting or non-secreting, columnar cells, with their nearly equal sex distribution and their increasing frequency with the advancing age, indicate that they are caused by comparatively weak carcinogenic agents, well established in the society, and acting with equal strength in both sexes, at least in large parts of the country.

The small group of bronchiolar cell carcinomas occur in all age groups and with no definite sex preference. They may be caused by unknown agents, hitting at random.

The histological pictures, the equal sex incidence, as well as the appearance with no preference for any age group, together indicate that lung adenomas, benign and malignant, and salivary gland tumours are caused by accidental factors, presumably of developmental origin.

At this stage a few more words about the latter tumours may be justified. Some surprise has been voiced as to the considerable number of adenomas and salivary gland tumours in Norwegian materials.

Firstly, a survey of the lung tumour problem in general in Norway shows that the total number of lung tumours is very moderate, as compared to the figures for England and Wales, the Netherlands, the United States, and even Denmark. If, as assumed and actually found, the recent increase in Norway is caused mainly by the ever larger number of Group I tumours, it follows that the other histological types must show a proportionate decrease in number. This development has been observed in the ordinary columnar cell carcinomas. A decrease in the number of lung adenomas and salivary gland tumours in relation to the sum total is predicted, but it may for a while be less marked in clinical and especially surgical materials, because these tumours represent comparatively favourable clinical cases and are therefore selected.

Secondly, the tumours in question are, actually, observed in considerable numbers also in other materials. Fried (1948) in the Massachusetts General Hospital found 17 adenomas out of 175 cases of bronchogenic neoplasms, and Carlens (1952) reported 8–10 per cent in a Swedish material.

Thirdly, these tumours are most probably present in many other materials without being recognized as such. True columnar cell carcinomas and Group I tumours very rarely occur before the age of 35 years. In most lung cancer statistics some tumours are registered in younger and very young persons. A closer study of the histology of these cases in our own material has revealed a con-

siderable number of lung adenomas as well as a few bronchiolar cell carcinomas. The youngest patient in the records of our Institute is represented by a boy, 12 years old, who suffered from an adenoma, and was reported upon by Harbitz (1937).

Fourthly, these tumours may actually have been seen in the microscope without being accepted as lung adenomas and salivary gland tumours. Some of them have been diagnosed as adenocarcinomas, others as small cell carcinomas, especially if unfavourable preparations have been the base for diagnosis, or if the tumours are infiltrating. Any academic discussion as to the propriety of the designation "malignant adenoma" ought to be silenced by the very important fact, that if the proper criteria are observed and the material typed without clinical information, a group of tumours will emerge, with a sex ratio 1:1, with no accumulation in any age group, and with an important number of cases in young persons. A philological discussion of the proper naming is of secondary interest. To close the eyes to these facts will only lead to the deprivation of some very interesting and important factors in the understanding of the lung tumour problem.

SUMMARY.

A material of 466 primary epithelial lung tumours have been histologically examined and typed. Each histological type has been analysed, especially as to distribution according to age and sex, and the findings correlated to our additional present knowledge regarding the development of these tumours. The following tentative conclusions have been drawn:

Squamous cell, large cell and small cell carcinomas are found predominantly in males. They do very seldom occur before the age of 35 years and they present a very characteristic and, for human carcinomas, singular age distribution. This age curve as well as the vital statistical evidence indicate that the main part of these tumours are caused by a recently established carcinogenic situation in certain parts of the world, notably the United States of America and Western Europe.

Columnar cell adenocarcinomas appear with little sex difference and they show a steadily increasing frequency with advancing age, a development in conformity with a great many human carcinomas. These tumours are probably caused by comparatively weak carcinogenic influences, evenly distributed over large areas, well established in the society and striking both sexes with equal force.

Bronchiolar cell carcinomas occur with equal frequency in both sexes and strike individuals in all ages. They are presumably caused by yet unknown factors, hitting at random.

Lung adenomas and salivary gland tumours in lungs, bronchi and trachea occur with the same frequency in both sexes and appear in all ages with accumulation in no age group. These factors as well as the histological picture indicate that these tumours are caused by chance developmental factors.

Lung "cancer" is accordingly histologically as well as biologically a heterogeneous group and this fact must be borne in mind when etiological factors are examined.

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I am likewise greatly indebted to Dr. Knut Westlund and Dr. Julie Backer for most valuable assistance during the preparation of the manuscript.

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THE OCCURRENCE OF LUNG CANCER IN NORWAY.

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THE picture of the development of lung cancer in Norway has hitherto solely been based upon the official mortality statistics, published yearly by " Statistisk Sentralbyrå," a service of the Norwegian Government.

As very few individuals have been cured from lung cancer, the mortality figures should give a fairly accurate account of the cases actually diagnosed and registered.

Up to and including the year 1940, a Scandinavian classification of causes of death was in use. The group " lung cancer " (no. 7027) included carcinomas and other malignant tumours, except sarcomas, of the lung and pleura. In 1941 the international classification of 1938 was introduced. The group " lung cancer " (no. 47*b*) was then to include only carcinomas of the lung and pleura. The omission of " other malignant tumours " made actually very little difference on the total number assigned to this group. The international classification of 1948 was introduced in 1951, and at present the group " lung cancer " includes the numbers 162 and 163. The most important change is the exclusion of tumours specified as secondary. This change in procedure has, however, not significantly influenced the figures for lung cancer mortality.

The tendency then, has been towards an increasing limitation of the term " lung cancer." But, even now it may be that certain tumours are differently coded in different countries. Especially might be mentioned the lung adenomas, benign and infiltrating, as well as some of the salivary gland tumours of the lungs. Histologically they are primary epithelial lung tumours. Clinically they often represent a " benign " tumour, and systematically most students would classify these tumours as benign. In a not unimportant number of cases they nevertheless behave like " lung cancer " and accordingly are classified as such. In the present Norwegian materials such tumours account for 10 to 15 per cent of all primary epithelial lung tumours.

Fig. 1 presents the absolute number of deaths from lung cancer in Norway from 1929 to 1952. In order to avoid the disturbance in comparability introduced by the 1948 classification, the figures for 1951 and 1952 have been adjusted, according to the analysis of Dr. Julie E. Backer, Head of the Medical Statistics Division of " Statistisk Sentralbyrå," so that they very approximately represent the figures that would have been found in group 47*b*, if the coding had taken place according to the 1938 classification. The figures are also given in Table I.

If we consider the twenty years period, from 1930 to 1950, and we take as the figures for the year 1930 the average of the years 1929–1931, we find for males a numerical increase from 15 to 150 cases, and for females from 11 to 66 cases This means a tenfold increase for males and a sixfold increase for females.

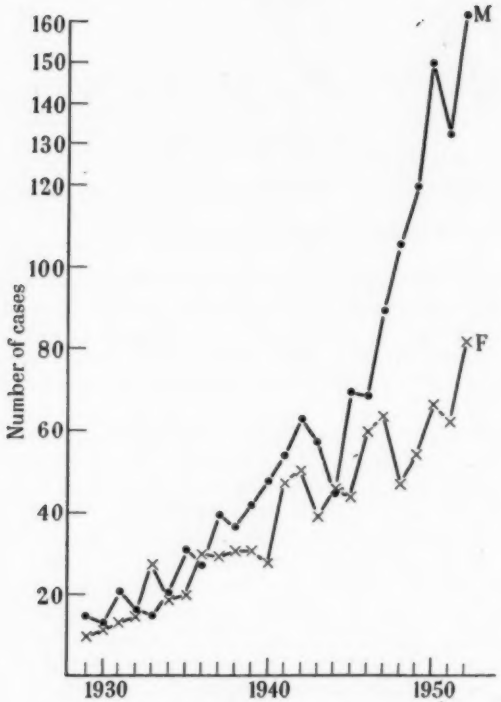

FIG. 1.—Lung cancer deaths in Norway, 1929–1952.

In the course of the twenty years' period the population has, however, increased considerably and the size of each age-group has changed.

Fig. 2 shows the lung cancer incidence adjusted to the age-distribution of the 1930 population. The pertinent figures are given in Table I. This graph repeats the tendency shown in the fig. 1, showing the gradual and parallel rise in lung cancer in both sexes up to the middle of the 1940's, however, with a slight lead of the males. From that period a marked change takes place. The number of cases diagnosed and registered in males increases more than that of the females.

The corrections introduced in fig. 2 lead, however, to a more accurate picture of the registered increase. In males this increase is from 0·11 per 10,000 living in 1930 to 0·77 in 1950. The corresponding figures for females are 0·075 in 1930 and 0·34 in 1950. Again the figures for 1930 are given as the mean value of the years 1929–1931. Instead of a tenfold increase in males we find a sevenfold, and instead of a sixfold increase in females, we find a four-and-a-half-fold.

In a recent paper Kreyberg (1954) has described 466 primary epithelial lung tumours and typed the tumours in six main types, according to criteria previously stated (Kreyberg, 1952). The total material consisted of two post mortem

TABLE I.—*Lung Cancer Deaths in Norway*, 1929–1952.

Year.	Number of deaths.		Total population.		Age—adjusted mortality per 10,000. (Standard : 1930).	
	M.	F.	M.	F.	M.	F.
1929	13	8	1,361,219	1,433,536	0·0978	0·0574
1930	12	11	1,368,169	1,439,269	0·0911	0·0769
1931	20	13	1,377,417	1,446,482	0·1427	0·0912
1932	16	15	1,387,435	1,454,131	0·1153	0·1030
1933	15	25	1,397,041	1,461,332	0·1071	0·1704
1934	20	18	1,406,123	1,468,110	0·1392	0·1201
1935	30	20	1,414,729	1,474,512	0·2079	0·1326
1936	26	29	1,422,952	1,480,590	0·1776	0·1900
1937	40	29	1,431,644	1,487,118	0·2707	0·1874
1938	35	30	1,441,191	1,494,633	0·1978	0·1911
1939	41	30	1,451,412	1,503,030	0·2729	0·1886
1940	46	27	1,461,361	1,511,728	0·2992	0·1673
1941	55	46	1,469,454	1,519,552	0·3466	0·2819
1942	63	51	1,477,938	1,528,432	0·3863	0·3007
1943	56	38	1,489,425	1,450,356	0·3358	0·2227
1944	45	47	1,503,333	1,554,071	0·2689	0·2727
1945	67	44	1,519,048	1,569,042	0·3917	0·2412
1946	66	58	1,537,951	1,586,298	0·3740	0·3225
1947	88	64	1,559,203	1,603,827	0·4780	0·3442
1948	106	45	1,577,632	1,621,368	0·5702	0·2390
1949	119	54	1,595,459	1,636,728	0·6320	0·2778
1950	150	66	1,618,324	1,645,664	0·7679	0·3387
1951	133	65	1,634,606	1,660,953	0·6860	0·3270
1952	161	82	—	—	—	—

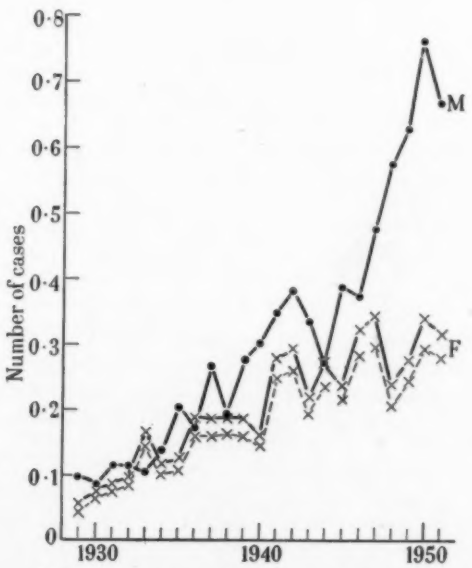

FIG. 2.—Mortality from "lung cancer" per 10,000—Norway. Standard figures, 1929–51. Two curves have been drawn for females, the upper giving the figures standardized to the female population in 1930, the lower the figures standardized to the male population of 1930.

series (Christiansen (1953), 134 cases, and Jakobsen (1953), 100 cases), as well as the authors own clinical material, embracing 232 cases.

The examination of the histological types, as well as the sex and age occurrence, in the light of our present clinical and epidemiological knowledge lead to the following conclusions: (i) Squamous cell, large-cell and small-cell carcinomas represent a biological entity. These tumours occur with a very marked preponderance in males, and they show an age-curve indicating that in the so-called Western World potent carcinogenic factors of recent origin are of aetiologic significance. These tumours are designated Group I tumours. (ii) Adenocarcinomas occur in Norway with the same frequency in males and females, and this tumour shows a steadily increasing occurrence with advancing years, indicating a stable and moderate carcinogenic influence. (iii) Bronchiolar cell carcinomas are likewise evenly distributed among males and females, but there is no increase in the higher age-groups. Unknown factors hitting at random are supposed responsible for their development. (iv) Lung adenomas and salivary gland tumours of the lungs show an equal sex distribution and occur in all ages. These facts, together with the histological picture point in the direction of developmental factors being responsible for their occurrence. These last four tumour types have been designated Group II tumours.

If the three Norwegian materials are examined and Christiansen's (1953) series is divided into two sub-series, according to two different time periods, we get the figures as shown in Table II. A few cases, which occurred in both Christiansen and Kreyberg's materials, have been included in the latter only in this table.

TABLE II.

Material.	Group I tumours.		Group II tumours.	
	Males.	Females.	Males.	Females.
Christiansen (1953) covering the years 1925–1944	25	11	15	13
Christiansen (1953) covering the years 1945–1952/1	40	4	14	12
Jakobsen (1953) covering the years 1937–1946	46	8	24	22
Kreyberg (1954) covering the years 1948–1953	162	8	28	29

Table II shows most strikingly the uniform occurrence of the Group II tumours in the two sexes in all the series, originating from different time periods. It should be emphasized that the present writer has been responsible for all the histological typing, and that the typing has been conducted at different periods and with no knowledge of the files, nor the clinical histories.

If the tentative conclusions presented above are correct, it would mean that the Group II tumours should represent primary lung tumours unrelated to special epidemiological circumstances, and that these tumours, with a possible reservation regarding the adenocarcinomas, should have the same relative frequency to-day as in the past. The adenocarcinomas may have increased in frequency in recent years, but the regular age-curve of this tumour type indicates that they have not very recently been influenced by external changes, in sharp contrast to the

Group I tumours. The consequence of this assumption should again be that an increase registered for Group II tumours would represent a measure of an increase in diagnostic efficacy.

As regards the Group I tumours, Table II again clearly reveals interesting facts. In the four time periods, the two middle partly overlapping, one may observe that females, in accord with clinical experience, constantly present a small number of Group I tumours. Compared now to the Group II tumours, they are at least not increasing in frequency. No conclusion in the opposite direction is drawn from the rather declining frequency because of the small figures.

It may therefore be correct to regard the lung cancer in females to-day as an expression of what Clemmesen, Nielsen and Jensen (1953) call " unavoidable " lung cancer. This unavoidable lung cancer should include all the Group II tumours, as well as a small number of Group I tumours.

If these assumptions are correct, the increase in lung cancer in females should at least in Norway today, be a fair index of the increased diagnostic efficacy. In the twenty years' period 1930–1950 this increase is approximately four-and-a-half-fold. We have, however, probably not yet reached the limitation of our diagnostic expansion. Although better diagnosis may in some cases lead to the discovery of a primary site outside the lung, and thereby exercise a depressive effect on the lung cancer mortality registered, it seems more likely that further improvements at the present time will mainly result in an increase in the mortality figures. We ought, therefore, to accept even a further increase in the lung cancer mortality registered in females, without necessarily drawing the conclusion that more lung cancer is actually developing. A very important clue as to a change in the situation is the ratio of the different histological types in the material.

If Table II and fig. 2 are correlated, one finds in males a different development taking place from the middle of the 1940's. From that period a marked increase in Group I tumours can be observed in males, at the same time as a pronounced deviation in the mortality rates in the two sexes takes place. It seems that the marked additional rise in lung tumours in males is caused solely by the Group I tumours. The Group II tumours, on the other hand, closely follow the pattern of the females. It may be that in the latest series (Kreyberg's) in Table II, the difference between the Group I and II tumours is too marked to be wholly representative, because that material is a clinical one with an underrepresentation of older people liable to develop adenocarcinomas. The tendency is clear, however, and the development continues to-day.

At present it seems permissible to conclude that in Norway, during the twenty years' period 1930–1950, the four-and-a-half-fold increase registered in lung cancer in females is entirely caused by greater diagnostic efficacy. As the development in males initially closely followed that of the females, it seems reasonable to accept the recent deviation in the mortality rates, as well as in the representation of the histological types, as indicating a new situation, and to regard the added increase in lung cancer in males as an expression of a real increase. This should mean the difference between a sevenfold and a four-and-a-half-fold increase.

The importance of this conclusion is not expressed by the magnitude of the real rise to-day, but it is indicated by the tendency and the short time it has been manifest. The increase in lung cancer in Norway has started later than in many other countries, but the pattern is identical.

SUMMARY.

The figures from the official Norwegian mortality statistics have been analysed on the background of the occurrence of different histological types of primary epithelial lung tumours in two Norwegian post-mortem materials and a series of clinical cases belonging to different time periods. It is found that in females the ratio of the different tumour types has not changed appreciably, and the increase in lung cancer in females is taken as indicator of the increased diagnostic efficacy. In males, on the other hand, a relative increase in the number of squamous cell, large-cell and small-cell carcinomas has taken place very recently, at the same time as a pronounced deviation in the mortality rates between the two sexes has developed. Accepting a four-and-a-half-fold increase in lung cancer in females as caused by better diagnosis, the difference between this figure and the sevenfold increase registered in males is taken to represent the real increase in the development of lung cancer in males.

The histological sub-division of the primary epithelial lung tumours into different histological and biological entities seems to be very useful in an analysis of the actual development of such tumours.

The present and the preceding paper were first read at the opening of our new Institute on March 16, 1953. Simultaneously, nearly identical observations and conclusions were in press in the important book ' Aetiologie und Prophylaxe des Lungenkrebses ' by F. Lickint (Dresden-Leipzig, 1953), unknown to the present author until our two papers were in press.

This study has been aided by a generous grant from " Tobaksfabrikernes Landsforening av 1901."

I am likewise greatly indepted to Dr. Knut Westlund for very valuable assistance during the preparation of the manuscript.

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THE ENDEMIOLOGY OF CANCER BY ORGAN SYSTEMS.

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In an age of high specialization there is a tendency to achieve understanding by the study of progressively smaller and still smaller units. In oncology this has taken the form of going from cancer as a whole to its various sites and types, and from tissues to cells to intracellular components and, finally, to molecules. Much progress has been made in this way in an understanding of the disease. This dissective analytical approach has, however, tended to divert attention from some of the larger units within individuals and in society which are still capable of teaching much about the natural history of the disease, including its etiology. The endemiological approach to etiology in cancer, which makes use of groups of cases, appears to be neglected.

The present purpose is to show by a statistical study of large bodily units, namely, organ systems, an endemiological pattern in the natural frequency of cancers that appears to have etiological implications. Each major body system shows a harmonious pattern in its principal tumours, different from other systems, suggesting that they have similar etiological factors.

In a recent statistical study, it was found that the curves for cancer to autopsy ratios by age were similar for all major sites in an organ system and different from those of other organ systems. The study was then extended to the mortality statistics of the United States, which corroborated the previous observations. It is believed that this behaviour pattern has etiological significance.

Necropsy Data.

The basic data from which the curves in Fig. 1 to 7 were constructed were obtained from the necropsy records of the Los Angeles County Hospital. The total number of autopsies in each decade is given in Fig. 1, and the number of cancers at each site can be found in the principal table of the appropriate chapters of another publication (Steiner, 1954). In that material of 35,293 necropsies, there were 6,072 malignant neoplasms. The twenty commonest cancers comprised 91·3 per cent of the total. Sixteen of the twenty (omitting carcinomas of the prostate, skin, pancreas, and the intracranial tumors because they are lone representatives of systems) fall into six organ systems. They form the basis for the present study.

If the curves of ratios of necropsies for cancer to total necropsies in the twenty tumors are plotted together, as in Fig. 1, a chaotic, apparently meaningless jumble is produced. Peaks are found at all ages. The relationships of the cancers appear haphazard and disorderly. However, if now the curves for each organ system are withdrawn from this melange and plotted separately, as in Fig. 2 to 7, an essential similarity is discovered for each system in that the configurations of

the component curves are similar, differing only in amplitude. An attractive order emerges from chaos.

Thus, the tumors of the four principal sites in the alimentary tract which give rise to carcinomas are plotted in Fig. 2. Carcinomas of the mouth, esophagus, stomach and large intestine had low mortalities early in life but they increased to a maximum in the seventh decade, after which their importance declined in relation to other causes of death. The four peaks are all at the same age, and the general configuration of the curves is the same, although they differ in quantity.

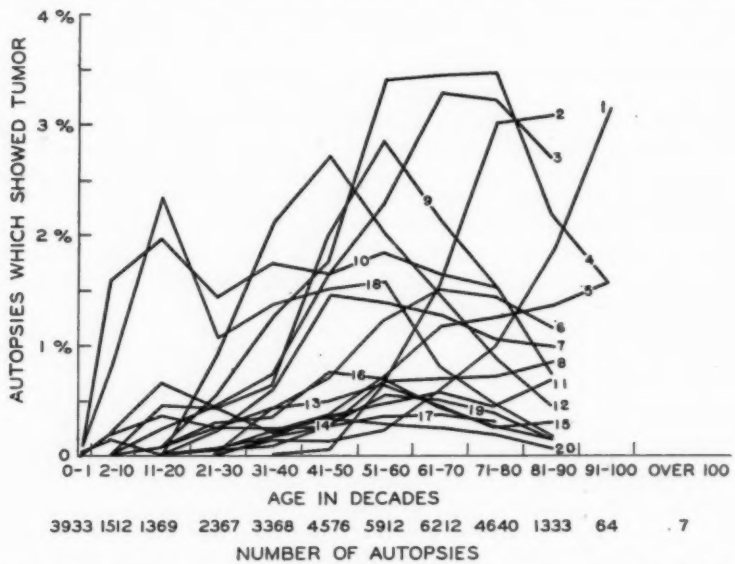

FIG. 1 to 7.—All curves show ratios (in per cent) of autopsies for cancers of specific sites found among all autopsies in each age period.

FIG. 1.—Shows that the cancer to autopsy ratios for the twenty commonest sites appear disorderly and chaotic. 1. Skin. 2. Prostate. 3. Large intestine. 4. Stomach. 5. Bladder. 6. Pancreas. 7. Breast. 8. Gallbladder. 9. Lung. 10. Lymphatic. 11. Kidney. 12. Uterus. 13. Mouth. 14. Esophagus. 15. Sarcoma (soft). 16. Ovary. 17. Liver. 18. Intracranial. 19. Larynx. 20. Bone.

If the carcinomas of the female generative organs are next abstracted from the mixture in Fig. 1 and plotted separately as in Fig. 3, they also have an essential similarity. The mortality from carcinomas of the mammary glands, uterus and ovaries began in the third decade, increased quickly as cancer to necropsy ratios to a maximum in the fifth decade, and then fell. The peaks and general configuration of the three curves are the same, differing only in amplitude. The mortality began soon after puberty, increased to the time of the menopause, and then fell off relative to other causes of death—more in the uterus and ovary than in breast. The peaks came two decades earlier than in the alimentary tract.

The curve for prostatic carcinoma is also plotted in Fig. 3 for contrast. Here appreciable mortality began in the sixth decade and increased thereafter. The

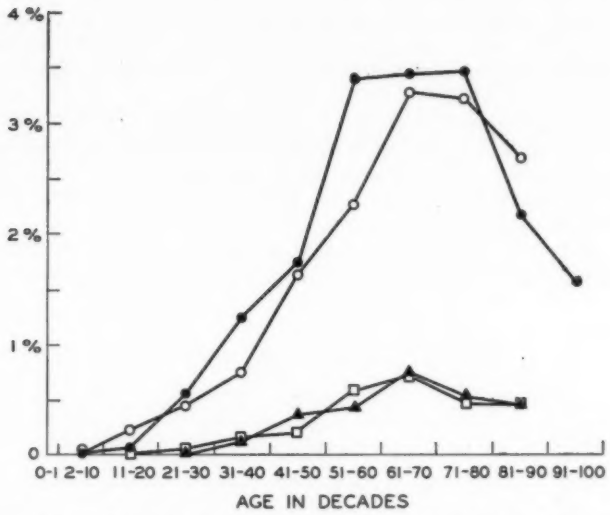

FIG. 2.—Shows order and similarity in the carcinoma to autopsy ratios in four sites of the alimentary tract separated from the melange of Fig. 1.

●——● Stomach. ▲——▲ Mouth.
○——○ Large intestine. □——□ Esophagus.

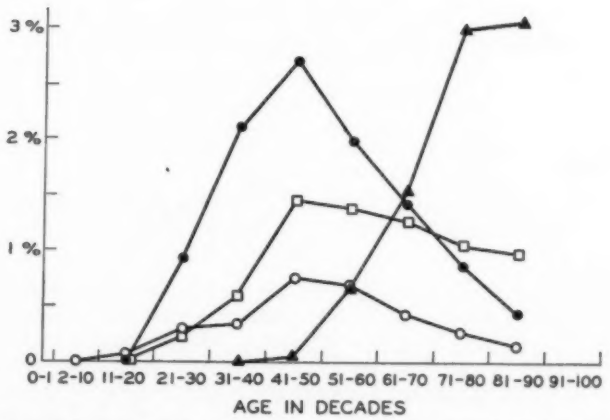

FIG. 3.—Similar carcinoma to autopsy ratios in the three tumours of the female generative system isolated from the apparent anrachy of Fig. 1. The highest ratios are in the fifth decades, two decades earlier than in the alimentary tract and four decades earlier than in the prostate.

●——● Uterus. ▲——▲ Prostate.
□——□ Breast. ⊙——⊙ Ovary.

peak was at least four decades later than in the female generative tract. Mortality began in the prostate when in the female generative organs it had already reached its peak. The curve for the prostate shows an inverse relationship to the period of most active sexual life, but a direct relationship is found in the female generative system.

The two principal tumours of the respiratory tract, free from the confusion of Fig. 1, are shown in Fig. 4. In carcinomas of the lung and larynx, also, the configuration is similar. The highest cancer necropsy ratios were found in the sixth decade—a decade later than in the female generative system and a decade earlier than in the alimentary tract.

In Fig. 5 the three principal neoplastic diseases of mesoblastic derivatives have been taken from the hodgepodge of Fig. 1 and plotted together. The sarco-

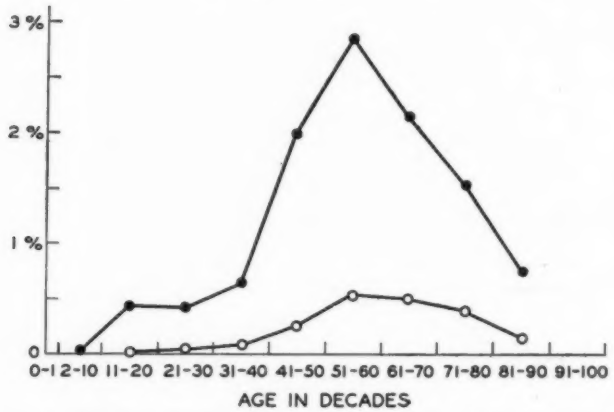

FIG. 4.—The carcinoma to autopsy ratios of the two sites in the respiratory tract show similar configuration.
●——● Lung. ○——○ Larynx.

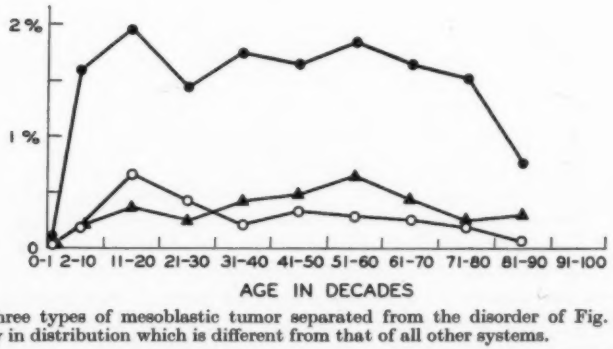

FIG. 5.—Three types of mesoblastic tumor separated from the disorder of Fig. 1, show a similarity in distribution which is different from that of all other systems.
●——● Lymphatic diseases. ▲——▲ Soft tissue sarcomas.
○——○ Bone tumors.

mas of the soft tissues and the bone tumors are remarkably similar with respect to cancer necropsy ratios and they differ from the malignant lymphatic diseases principally in being fewer. In all three varieties there was a virtual plateau from the first to the eight decades. This configuration is entirely different from that found in any other system except for the intracranial tumours. As depicted here, the lymphatic disease category is a composite of the leukemias, aleukemias, lymphosarcomas and malignant lymphogranulomas.

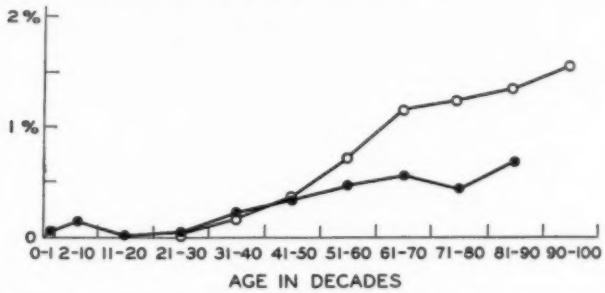

FIG. 6.—Carcinoma to autopsy ratios at the two sites in the urinary tract. The configuration is different from any of the preceding curves.

○———○ Bladder. ●———● Kidney.

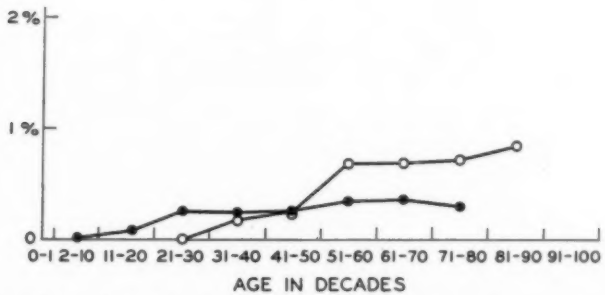

FIG. 7.—Shows the carcinoma to autopsy ratios in the biliary system.

○———○ Gall bladder. ●———● Liver.

The two principal carcinomas of the urinary tract, namely, bladder and kidney, are shown in Fig. 6, freed from the disorder of Fig. 1. Except for the small peak in renal cancers in the first decade, caused by the Wilm's tumors, the configuration of the curves is the same. There was a gradual increase with age in these cancer necropsy ratios. The curves differ in their configuration from those of the previously illustrated systems.

The two principal carcinomas of the biliary system in this material are shown in Fig. 7. Although mortality from carcinoma of the liver began earlier than from the gall-bladder, in both it increased with age, and there is a similarity in the curves which is not obvious in the apparent anarchy in Fig. 1.

Mortality Data.

That the necropsy data used in the previous section are not unique in their endemiological behavior is shown by a comparison with the U.S. mortality figures. In the Vital Statistics for 1950, 210,733 fatal malignant neoplasms were reported among 1,452,454 deaths from all causes. The terminology and classification is different from that used in the Los Angeles material, despite which the comparisons that follow can be made. In most organs the sarcomas are classed with the carcinomas. The cancers of the stomach, intestine (including rectum), esophagus and the buccal cavity (including the pharynx) had totals of 24,258, 33,383, 3,866 and 5,138 cases respectively. If cancer death to all death ratios are made by decenniums or by quinquenniums, the configurations of the curves are the same for all four tumours, and they resemble those in Fig. 2. The cancers of the buccal cavity show a virtual plateau in the two decades 50–59 and 60–69.

In the respiratory tract, 18,277 cancers were reported in the trachea, bronchi and lung (some unspecified as primary or secondary) and 1,852 in the larynx. The cancer to death ratios show peaks in the decade 50–59 as in Fig. 4. These peaks, also, lie between those found in the alimentary tract and the female generative organs.

Similarly, the three principal cancers of the female generative organs (mammary glands, uterus and ovary plus Fallopian tube and broad ligament) show curves which are similar in configuration to each other and to those in Fig. 3, with peaks in the decade 40–49. There were reported in 1950 18,973 cases of cancer of the breast, 16,085 of the uterus, and 5,652 of the ovary, Fallopian tube and broad ligament combined.

In the mesoblastic series of malignant tumours, those of bone (2,180 cases) showed a curve resembling that in Fig. 5 with a peak in the decade 10–19, a small depression in the decade 30–39, and a small secondary rise followed by a gradual decline. The malignant lymphatic diseases (code numbers 200–205; total of 16,756 cases) showed a peak in the first decade followed by a gradual decline. The ratios to all deaths were higher during the first three decades than in the Los Angeles material. The sarcomas of all soft tissues are not combined in the U.S. Vital Statistics, so this category cannot be studied.

In the urinary tract, kidney tumors (3,643 cases) had a maximum in the decade 50–59 while bladder tumors (6,401 cases) had a peak a decade later. Neither curve showed the persistent rise of Fig. 6 in the older age groups.

Because all tumors of the liver and biliary passages are combined in the U.S. Vital Statistics, comparisons cannot be made between individual sites or with Fig. 7.

The U.S. Vital Statistics, in summary, in general confirm the observations made in the Los Angeles necropsies that the malignant tumors of the different parts of each system have cancer to death ratios which form curves of similar configuration although differing in quantity. It is deduced, therefore, that the Los Angeles necropsies were not unbalanced, biased, or highly selected in this regard, and that the principle has wider application.

DISCUSSION.

It is seen that order exists in the apparent chaos of Fig. 1. Within each of six organ systems, the tumors show a similarity in their statistical behavior as

shown in Fig. 2 to 7. Furthermore, each system is different from the others, except for the urinary and biliary tracts. Instead of anarchy, there emerges a statistical harmony. There is no exception to this rule in the tumors of the six major systems studied.

In so far as comparisons are possible the U.S. mortality figures for 1950 conform to, with minor exceptions, the same endemiological patterns as the Los Angeles necropsies, showing that the latter were not exceptional in this regard.

It should be noted that cancer to autopsy ratios were used in one set of data and cancer to death ratios in the other. Thus, autopsies for all causes and all deaths were used, respectively, as the points of reference. Cancer to autopsy ratios may be of limited value in expressing total quantity but they are excellent for contrasting one site with another within a material because the point of reference within each age group is the same for all. However, essentially the same endemiological facts are revealed by other methods of analysis, although less clearly. Thus, the age distribution figures (Steiner, 1954) for the cases in the Los Angeles necropsies show similarities within organ systems. The differences between systems exist but they are less apparent than are the cancer to necropsy ratios used here. True incidence rate figures gave similar results in so far as they were compared. Thus, if the data of Clemmesen and Nielsen (1952) from the Danish Cancer Registry are used, the incidence curves for cancers of the stomach, esophagus and large intestine (WHO code 153.0–3, 153.9 plus 154) are similar in configuration, as are those of the uterus and ovary but not of the mammary gland.

The question arises as to what, if anything, these statistical findings indicate regarding etiology. Are they explained by chance? That they probably are not is shown by the absence of exception in sixteen tumor sites representing six systems in the Los Angeles necropsy series, and an essential concordance by most of thirteen tumors in five systems in the U.S. mortality data. Such a high degree of agreement is hardly chance.

If they are not chance phenomena, what do they signify ? Could they indicate common etiological factors within systems but different between systems ? The principal groups of etiological factors at this time appear to be (*a*) those that govern the susceptibility of the cell to undergo cancerous transformation and (*b*) those that act on cells to induce this change.

Established principles in experimental carcinogenesis may pertain to the endemiological situation here demonstrated in man. It is readily apparent from Fig. 2 to 7 that the organs in some systems develop a greater proportion of their cancers at an earlier age than in others. It is not known whether this earlier occurrence represents an earlier exposure to etiological factors or a shorter tumor induction time after the beginning of the exposure. Induction time is the resultant of many factors including type, potency and amount of carcinogen, number of carcinogens, conditions of exposure and susceptibility of the cells. That differences in induction time exist in man is indicated by the exposures to ionizing radiation in Japan (Hiroshima and Nagasaki) where to date only leukemias have occurred although ionizing radiations are known to induce tumors of other types.

It is also apparent from the figures that the tumor yield by sites within systems varies greatly although the induction time appears to be the same. Tumor yield is governed, in general, by the same factors as the induction time just mentioned. In addition the number of cells being exposed is important in determining the number of tumors obtained. The number of tumors observed in each part of an

organ system can, in theory, be correlated in part with the number of cells at each site exposed to hypothetical carcinogens. Thus, in the alimentary tract there would appear to be, on the basis of the gross and microscopical anatomy, approximately equal numbers of epithelial cells available for neoplastic conversion in the mouth and esophagus, many more in the stomach, and still more in the large intestine. This is roughly parallel to the numbers of cancers that occur in these locations (in 1950 5,138, 3,866, 24,258 and 33,383 respectively in the United States). Similar agreements between the relative numbers of epithelial cells and tumors are found in the larynx and lung, in the female generative organs (if one assumes that most mammary carcinomas are ductal), and in the mesoblastic tissues if nuclei rather than total volume are considered. The correlation fails in the biliary system, the small intestine and the urinary tract except if it is assumed that not all cells are exposed. Local factors may interfere with the actual exposure of cells. In the absence of such an explanation, the idea of site differences in cell susceptibility receives support.

In the past it has been customary to explain the quantitative differences in site distribution of cancers within systems on the basis of local susceptibility or resistance factors. It is here suggested that the quantity of the cell populations at risk needs also to be considered.

SUMMARY.

An endemiological study was made of the malignant neoplasms at sixteen sites in six organ systems in 35,293 autopsies at the Los Angeles County Hospital. Curves were made of ratios of necropsies for malignant neoplasm to all necropsies by age.

The cancer to necropsy ratios for the tumors of the different sites within each organ system showed similar curves; they differed from each other only in amplitude. Furthermore, the curves for each system were characteristic and they tended to differ from those of other systems.

Similar results were obtained by an analysis of the U.S. mortality statistics for 1950. The curves of cancer to total mortality ratios by age in thirteen common tumors found in five systems showed a harmonious behavior within each system, which differed from that of other systems.

Some case distribution and some incidence data show the same statistical facts although less clearly.

The etiological significance of these endemiological observations is unknown. However, it is suggested that they could be interpreted as signifying common etiological factors within each system with the differences in quantity in tumor by sites accounted for in part by the unequal numbers of cell populations at risk. Comments are made about the interpretation of the quantitative differences in tumors between organ systems and in their age distribution.

The author is grateful to the Research Committee of the Los Angeles County Hospital Staff for permission to study their material and to Dr. E. M. Butt for his interest and help.

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CANCER AMONG CANADIAN INDIANS.

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WITHIN recent years evidence has been brought forth on variations in the distribution of cancer in different parts of the world. Cancer of the base of the tongue, for example, is relatively frequent in certain parts of India and primary cancer of the liver is relatively frequent in Africa and Indonesia. Cancer of the stomach on the other hand, is relatively infrequent in African Negroes, Javanese and the indigenous people of French North Africa. Such observations have focused attention upon racial origin, environment and custom as important factors in the study of cancer.

In 1950 at a conference on the geographical pathology and demography of cancer (Clemmesen, 1950*a*) it was agreed that a high degree of comparability is desirable in any study of geographical variation. The conference recommended that each study should provide :

(*a*) The total number of new cases in the area being studied.
(*b*) Distribution of the total population and of patients in the area with respect to race, sex and age.
(*c*) Percentage of cases diagnosed in hospitals.
(*d*) Percentage of cases diagnosed by histological examination.
(*e*) Percentage of cases verified by autopsy.
(*f*) Description of any subdivision undertaken (social, occupational, geographical, etc.).
(*g*) Statement of the type and location of hospitals from which statistics have been gathered.

The authors have studied the Indian population of Canada believing that a contribution might be made to the data already assembled on the endemiology of cancer. The group offers a unique opportunity for study since data are available on each of the above-mentioned points.

In 1936 Palmer reported " that cancer is less frequent among the Indians than among other races living in the United States and Canada."

This general conclusion upon the Canadian Indians was drawn from communications with members of the Department of Indian Affairs in Canada. In 1928 Dr. L. L. Stowe of the Indian Medical Service stated that " as far as my observations and enquiry justify a conclusion, the disease is very rare," and in 1936 the Deputy Superintendent General of the Department of Indian Affairs replied to a questionnaire from Palmer with the statement that " This disease occurs so infrequently among Indians in Canada that its incidence is about negligible."

The opinions expressed at these times were of necessity based upon personal observations and enquiry, because complete data from across Canada were not then available for study.

The origin of the North American Indian remains uncertain, but anthropologists believe that they came to America in successive migrations in prehistoric times from northern Asia, probably by way of the Bering Sea. Today the Canadian Indian is divided into a number of basic language groups with differing physical and psychological characteristics. There are nearly six hundred separate Indian communities known as " bands." Except for certain nomadic groups inhabiting the outlying and northern regions, these bands are located on more than 2200 " reserves." These " reserves " vary in size from a few acres to more than 500 square miles and have been set aside by the Canadian Government for the use and benefit of the Indians.

It has been estimated that at the time of the first settlements in North America, about four centuries ago, the Indian population of what is now Canada was approximately 200,000. Shortly after the advent of Europeans the number of Indians began to decline and it became a common belief that the Indians were a dying race. In the last half century, however, there has been a steady increase and today this population group is growing at the rate of 1·5 per cent annually. The Census of Indians in Canada in 1949 (Department of Citizenship and Immigration, 1949), showed a total population of 136,407, of which 69,593 were males and 66,814 females.

By a special provision in the British North America Act of 1867 the administration of Indian affairs, which had been under the management of several provinces, came under the jurisdiction of the Government of Canada. At the present time Indian affairs are the responsibility of a branch of the Department of Citizenship and Immigration. The primary function of this branch is to administer the affairs of the Indians of Canada in a manner that will enable them to become increasingly self-supporting and independent members of the community.

Health services to Indians date back to the early part of the seventeenth century when French army doctors gave medical care to this group. The first attempt at a government sponsored health plan was made in 1905 when doctors and hospitalization were made available to all Indians within reach of transportation. This programme was extended in 1922 by the appointment of field nurses. In 1927 a full-time superintendent of Indian medical services was appointed. Staff headquarters are located in Ottawa under the Department of National Health and Welfare. The Indian Health Service operates 18 hospitals and 29 nursing stations giving a capacity of approximately 2287 beds. Across the country there are 65 full-time doctors caring for Indians. There are also 90 public health nurses assisted by provincial public health nurses, Red Cross outpost nurses and the Victorian Order of Nurses. Centres range in size from the 456-bed Charles Camsell Indian Hospital at Edmonton, Alberta, to small units of 16 to 20 beds. In addition the services of some 380 community and private hospitals are utilized for the treatment of Indians.

The Indian Health Service has been faced with a number of problems of which tuberculosis, inadequate nutrition and high infant mortality are the most serious. As might be expected the standard of pre- and post-natal care is affected by the wide distribution of the population and the limited services available. In the early 1930's budgetary restrictions limited the number of deliveries in hospital but since then increasing efforts have been made to encourage prospective mothers to come to medical centres for delivery.

The total Indian population constitutes approximately one per cent of the

population of Canada.* A comparison of birth-rates, infant mortality rates and general mortality rates between Indians and Whites is shown in Table I for the year 1949 (Department of Trade and Commerce, 1949). It will be noted that the birth rate among Indians is double and that the infant mortality rate is approximately three times that in the general population. The general mortality rate is more than double.

TABLE I.—*Comparison of Birth Rates, Infant Mortality Rates and General Mortality Rates Between Indians and Whites.*

	Rates per 1000.	
	Indians	Whites.
Birth rate	55·4	27·1
Infant mortality	128·8	43·3
General mortality rate	21·4	9·1

The leading causes of death differ for Indians and Whites as shown in Table II. When making comparisons it is to be noted that the death rate for ill-defined causes is relatively high.

TABLE II.—*Mortality Rates in Certain Categories for Indians and Whites in Canada, 1949.*

Cause of death.	Mortality rate per 100,000 population.	
	Indians	Whites
Tuberculosis	439·1	31·4
Cancer	68·9	123·7
Diseases of nervous system	98·2	93·2
Diseases of the heart	160·5	262·7
Diseases of respiratory system	335·0	54·7
Diseases of digestive system	177·4	47·7
Ill-defined causes including senility	244·1	18·6
All causes	2139·2	917·2

In any analysis of variations between two ethnic groups it is essential to know the age structure of each population. Table III shows the proportion of Indians and Whites by decennial age groups. The age structure of these populations varies considerably.

TABLE III.—*Proportion of Indians and Whites in Decennial Age Groups (1951 Census).*

Age group.	Indian (per cent).		White (per cent).	
19 years and under	53·3		37·9	
20–29	15·1	79·1	15·8	68·3
30–39	10·7		14·6	
40–49	8·1		11·5	
50–59	5·9	20·9	8·8	31·7
60–69	3·7		6·7	
70 and over	3·2		4·7	
	100·0		100·0	

* Census population of Canada, 1951, 13,984,400 persons (exclusive of Yukon and North-West Territories, 24,000 persons).

When an Indian receives medical care a record of his case is forwarded to the national office of the Indian Health Service. This record gives, among other data, the patient's name, band number, age, sex, diagnosis, and days of hospitalization. With such information available the patient-records in forty-eight hospitals were reviewed for the years 1948–1952 inclusive and all cases of cancer were selected for analysis. The Indian Health Service has estimated that over 90 per cent of Indians with cancer would be referred to the hospitals included in this study.

In the five years which were reviewed, 327 cases of cancer were reported, 125 in males and 202 in females. Among the males one half of the cases occured in two main sites—the digestive and the urinary systems. Among the females cancer of the cervix accounted for approximately 40 per cent of all cases. TableIV shows the distribution of cases by site and sex. In 77 per cent there was histological proof of diagnosis, a figure which compares favourably with the studies of Watson (1950) 78 per cent, Macdonald (1948) 74 per cent, and Dorn (1944), 68 per cent.

TABLE IV.—*Cancer Cases among Indians by Site and Sex* (1948–1952).

Site.	Number of cases. Male.	Female.	Total.	Pathologically proved.
Buccal cavity	13	7	20	20
Digestive system	32	20	52	31
Respiratory system	10	5	15	11
Breast	0	26	26	19
Cervix	0	83	83	74
Uterus, ovary, vulva	0	22	22	19
Urinary organs	31	4	35	22
Skin	11	16	27	22
Haemic and lymphatic	11	5	16	16
Bone	2	2	4	2
Brain	3	3	6	3
Other sites	9	7	16	11
Primary unknown	3	2	5	2
All sites	125	202	327	252 (77%)

From the data gathered the total incidence of cancer among Indians is 48·4 per 100,000 as compared with 203 by Watson (1950), 207·8 by Macdonald (1948), and 230 by Dorn (1944). Fig. I shows the incidence of cancer by age among Indians as compared with that reported by Watson (1950) and Dorn (1944).

The data have been examined by means of the " chi-square " test after the model given by Fisher (1946). Table V shows the results of this analysis.

All values of chi-square are significant at the 1 per cent level except for males (20–29) and females (under 20 years, and 20–29 years).

It may be concluded that the apparent incidence of cancer among Indians is significantly less than that among Whites except in these mentioned age groups.

The chi-square test has also been applied to differences in incidence by site. Table VI shows the distribution of 327 Indian cancer cases according to the most common sites. Also shown are the number of cases for each site which might be expected to occur in this group if the incidence by site were the same as in the population studied by Watson (1950). This analysis produces two chi-square values which are highly significant, one for skin (30·19) and one for cervix (638·15). It will be noted that these sites vary in opposite directions ; the observed number

of skin cases being less than the expected number while the observed number of cases of the cervix uteri was greater than the expected number. When these two sites were deleted from the analysis it was found that the chi-square for the remainder was not significant, hence we conclude that only in the incidence of cancer of the skin and cervix do the Indians differ from the Whites.

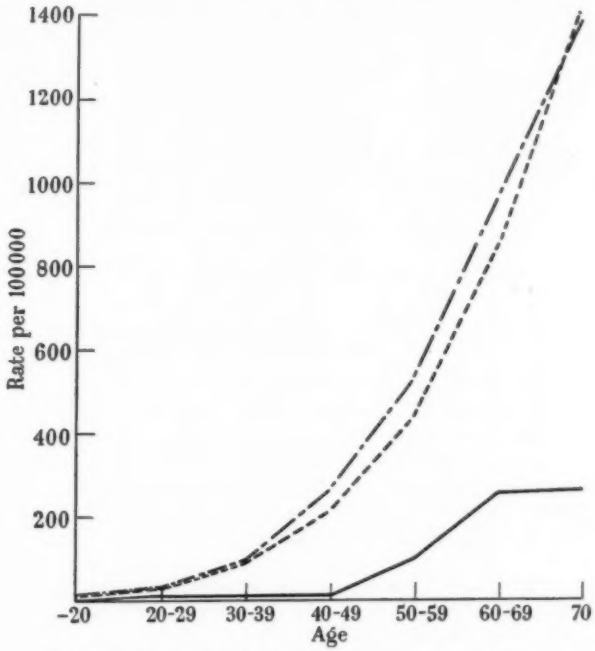

FIG. 1.—The incidence of cancer in Indians and Whites.
——— Indians. — — — Dorn (1944) - - - - Watson (1950).

TABLE V.—*Application of "Chi-square" Test to Age Incidence of Cancer among Indians.*

Age.	Male				Female			
	Observed freq.	Expected freq.	Pop. of age group.	χ^2.	Observed freq.	Expected freq.	Pop. of age group.	χ^2.
Under 20	7	19	41,296	7·58	7	14	41,692	3·50
20–29	8	13	11,770	1·16	12	18	11,640	2·00
30–39	6	22	8,660	11·65	32	53	8,045	8·37
40–49	17	58	6,819	29·23	46	103	5,891	32·10
50–59	16	120	5,064	92·32	31	123	4,167	70·90
60–69	36	162	3,191	103·24	39	119	2,601	56·36
70 plus	31	207	2,542	162·91	31	145	2,494	95·16
Total	121*	601	79,342	386·29	198*	575	76,530	249·05

* Ages of 4 males and 4 females unknown.

The observed difference in the incidence of cancer of the cervix uteri is so marked that it has been investigated further. Fig. 2 shows the age distribution of the Indian cases compared with those from the Saskatoon and Regina cancer clinics in Saskatchewan and the Ontario Institute of Radiotherapy at the Toronto General Hospital in Toronto, Ontario. In each instance the five-year period, 1948–1952, has been considered. It would appear from this analysis that cancer of the cervix has a tendency to occur earlier in life among Indians than it does among Whites.

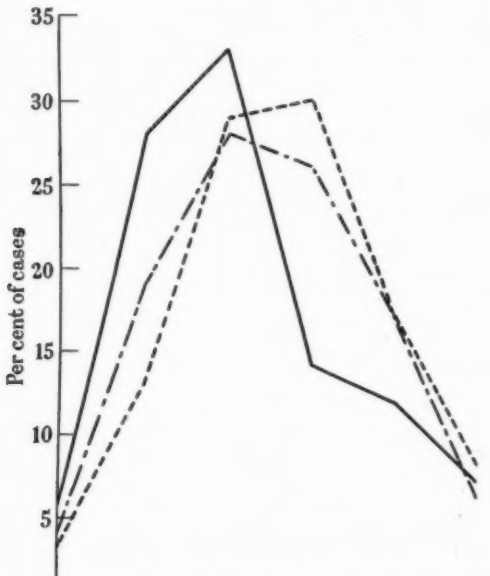

FIG. 2.—Age distribution of cancer of cervix cases.
——— Indians. — — — Toronto General Hospital.
– – – – Saskatchewan Clinics.

DISCUSSION.

The significant facts arising from this study of the incidence of cancer among Indians are :

(*a*) Cancer among Canadian Indians is not so uncommon as has been reported previously, but the total incidence is apparently less than in Whites.

(*b*) Under 30 years of age there is no difference in the incidence of the disease between Indians and Whites.

(*c*) Over the age of 30 the incidence of cancer is apparently less among Indians.

TABLE VI.—*Application of "Chi-square" Test to Site Incidence of Cancer among Indians.*

Site.	Observed frequency.	Expected frequency.*	χ^2.
Buccal cavity	20	31·84	4·40
Digestive system	52	71·78	5·45
Respiratory system	15	16·58	0·15
Breast	26	25·99	0·00
Cervix	83	8·66	638·15
Uterus	22	16·20	2·07
Urinary	35	34·46	0·01
Skin	27	74·39	30·19
Haemic and lymphatic	16	11·87	1·44
Bone	4	5·09	0·23
Brain	6	6·59	0·05
Other	21	23·55	0·28
Total	327	327·00	682·42

* Based on incidence rates by sites in Watson (1950).

(*d*) The incidence of cancer of the skin is apparently less among Indians, and

(*e*) The incidence of cancer of the cervix is much greater among Indians and the disease tends to occur earlier.

The first two of these observations require no discussion. The finding that cancer is less frequent in Indians over 30 years of age than in Whites over 30 years of age is to be accepted with caution. "Ill-defined causes" are reported in death certification more frequently (Table II) for Indians and it is possible that illness and deaths from cancer may lie within this group and which, if properly reported, would raise the incidence rates for older Indians. In other words, the observed difference in total cancer incidence rates between Indians and Whites may be more apparent than real.

It is possible that skin pigmentation of Indians acts as a protection against skin cancer in the same way as for the black race. However, it is also possible that skin cancer cases did not come under medical care but we doubt this because the incidence of other sites, excepting cervix, was the same as in the White population.

The most interesting finding is that relating to the high incidence of cancer of the cervix uteri. The explanation remains unknown. It is doubtful if this is to be found in any error of statistical approach because of the similarity in incidence rates for other sites of the disease. The high birth rate and limited post-natal care may be related factors. Clinical studies are being undertaken to investigate the high incidence of cancer of the cervix in Indian women.

SUMMARY.

A statistical study has been made of the incidence of cancer among Canadian Indians.

The rates have been compared with those already reported for the White population of Canada and the United States.

Under the age of 30 years there is no difference in the incidence of the disease between the two groups.

Over this age the incidence of cancer among Indians is apparently less than among Whites.

Cancer of the skin occurs less frequently among Indians.

The incidence of cancer of the cervix uteri is much higher among Indians than that reported for the White population in Canada and the disease tends to occur earlier in life.

The incidence rates for all other sites of the disease show no difference between Indians and Whites.

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GASTRO-INTESTINAL CANCER AND THE USE OF LIQUID PARAFFIN.

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FROM data collected in a previous inquiry (Doll and Hill, 1950) it was possible to make some investigation of the past use of purgatives of a group of patients suffering from cancer of the gastro-intestinal tract and to compare this experience with that of a large number of patients whose disease was other than gastro-intestinal cancer.

Purgative histories were available for 2249 patients. For these patients information had been obtained about whether purgatives were used, "Often" (*i.e.*, regularly at least once per week), "occasionally" (*i.e.*, regularly at least once per month) or not at all; about the age at starting the use of purgatives, the age at stopping the use of purgatives, and about the type of purgative used. For the purpose of the analysis only patients whose history of purgative taking extended over a continuous period of at least 5 years were considered as "purgative users." This arbitrary definition of "purgative user" was set up to exclude sporadic users of purgatives and those patients who had started to take purgatives as a result of the symptoms produced by cancer in the gastro-intestinal tract. Three hundred and twenty-two patients who were suffering from gastro-intestinal disease other than cancer were excluded from the analysis since, in a large proportion of this group (*e.g.*, the many peptic ulcer patients), their disease was already of more than 5 years' duration at the time of interview and may well have given rise to the formation of the purgative habit.

The data remaining for analysis consisted of purgative histories of 1927 patients, of whom 614 had been diagnosed as having a gastro-intestinal cancer and 1313 formed a control group of non-gastro-intestinal patients. Of the 614 gastro-intestinal cancers, 387 were cancers of the large bowel and 227 were cancers of the stomach: the control group consisted of 647 patients with cancers of the lung and 666 patients with non-gastro-intestinal diseases other than cancer.

RESULTS.

The combined purgative experience of the gastro-intestinal cancer patients and the non-gastro-intestinal patients was tabulated in four age-groupings for males and females separately (Table I), and utilised to obtain the purgative consumption "expected" if both groups of patients had been subject to the same overall purgative rates (Table II). Thus for the 34 male gastro-intestinal cancer patients under 45 years of age, one would expect $\frac{112}{177} \times 34$ or 21·5 to have never

TABLE I.—*Combined Purgative Histories of Patients Suffering from Gastro-intestinal Cancer and Non-gastro-intestinal Disease (by Age and Sex).*

Age (years).	Men. Use of Purgatives. Never.	Occasionally.	Often.	Total cases.	Women. Use of purgatives. Never.	Occasionally.	Often.	Total cases.
Under 45	112	33	32	177	39	12	18	69
45–	277	70	103	450	56	21	36	113
55–	296	104	150	550	39	21	56	116
65+	155	63	106	324	47	17	64	128
All ages	840	270	391	1501	181	71	174	426

TABLE II.—*Purgative Histories of Gastro-intestinal Cancer Patients (by Age and Sex).*

Age (years).		Men. Use of purgatives. Never.	Occasionally.	Often.	Total cases.	Women. Use of purgatives. Never.	Occasionally.	Often.	Total cases.
Under 45:	Obs.	19	4	11	34	17	4	8	29
45–	Obs.	41	11	17	69	29	10	19	58
55–	Obs.	67	27	47	141	25	14	34	73
65+	Obs.	60	23	37	120	28	13	49	90
All ages:	Obs.	187	65	112	364	99	41	110	250
	Exp.	197·3	67·0	99·7	364·0	102·6	41·0	106·3	249·9

used purgatives; $\frac{33}{177} \times 34$ or 6·3 to have used purgatives occasionally; and $\frac{32}{177} \times 34$ or 6·1 to have taken purgatives often. In a like manner the "expected" purgative consumption was calculated separately for each age-group in each sex. By adding the experience of all age-groups in each sex and then combining the sexes an estimate was thus obtained of the "expected" purgative consumption, in which appropriate allowance had been made for for the age and sex composition of the gastro-intestinal cancer group. The "expected" purgative consumption for non-gastro-intestinal patients was calculated by the same method. On comparison of the two groups (Table III) there appeared to be an

TABLE III.—*Comparison of Purgative Histories after Standardisation for Age and Sex (all Persons).*

Disease group.		Use of purgatives. Never.	Occasionally.	Often.	Total cases.
Gastro-intestinal cancer:	Obs.	286	106	222	614
	Exp.	299·9	108·0	206·0	613·9
Diseases other than gastro-intestinal cancer:	Obs.	735	235	343	1313
	Exp.	721·1	233·0	359·0	1313·1

$\chi^2 = 2{\cdot}92$; $n = 2$; $P > 0{\cdot}1$.

excess of heavier users of purgatives amongst gastro-intestinal cancer patients (*e.g.*, 222 patients in this group gave histories of having taken purgatives "often," while the "expected" number was 206). The differences, however, were such as might well be due to chance alone. A similar trend towards heavier usage was found for both cancer of the large bowel and cancer of the stomach when these groups were considered separately, being more marked for male patients with cancer of the large bowel and for females with cancer of the stomach. Again however, the differences noted—while consistent and in the same direction—did not reach a statistically significant level.

Following the comparison of overall purgative usage, an attempt was made to discover whether the experience of the gastro-intestinal cancer and non-gastro-intestinal groups differed when specific purgatives were considered separately. As a first step crude rates expressing the usage of 14 purgatives were calculated for the four disease groups: cancer of the large bowel, cancer of the stomach, cancer of the lung, and non-gastro-intestinal diseases other than cancer (Table IV). Study of these rates suggested that for only three purgatives—liquid

TABLE IV.—*Proportion of Patients Using Specific Purgatives in each of Four Disease Groups.*

Purgative.	Disease group. Cancer of large bowel.		Cancer of stomach.		Cancer of lung.		Non-gastro-intestinal disease other than cancer.	
	No.	%	No.	%	No.	%	No.	%
Cascara	26	6·7	11	4·8	31	4·8	40	6·0
Senna	23	5·9	15	6·6	13	2·0	21	3·2
Beecham's pills	39	10·1	21	9·3	28	4·3	34	5·1
Salts	108	27·9	60	26·4	165	25·5	168	25·2
Liquid paraffin	45	11·6	24	10·6	23	3·5	35	5·3
Bile beans	10	2·6	2	0·9	8	1·2	11	1·7
Syrup of figs	14	1·0	2	0·9	3	0·5	3	0·5
Chocolate laxative	9	2·3	5	2·2	14	2·2	7	1·1
Vegetable laxative	10	2·6	2	0·9	8	1·2	9	1·4
Castor oil	3	0·8	1	0·4	0	0	4	0·6
Liver pills	8	2·1	6	2·6	14	2·2	6	0·9
Liquorice powder	3	0·8	6	2·6	2	0·3	7	1·1
Brimstone and treacle	2	0·5	3	1·3	1	0·2	0	0
Seidlitz powder	8	2·1	3	1·3	2	0·3	3	0·5
Total No. of patients in each disease group	387	(100%)	227	(100%)	647	(100%)	666	(100%)

paraffin, senna, and Beecham's pills—was there a marked and consistent difference between the gastro-intestinal cancer and non-gastro-intestinal groups. Since, however, these rates were calculated without due allowance for the differing age and sex composition of the populations involved, further analysis of the data on these three purgatives was required.

Age and sex specific usage rates of liquid paraffin, senna and Beecham's pills were calculated for gastro-intestinal cancer and for non-gastro-intestinal patients (Table V). The differences in the liquid paraffin rates were the most

TABLE V.—*Purgative Usage of Patients with Gastro-intestinal Cancer and of Patients with Non-gastro-intestinal Disease. (Age Specific Rates (%) for Use of Liquid Paraffin, Senna, and Beecham's Pills.*

Sex.	Age (years).	Purgative. Liquid paraffin. Gastro-intestinal cancer.	Liquid paraffin. Non-gastro-intestinal disease.	Senna. Gastro-intestinal cancer.	Senna. Non-gastro-intestinal disease.	Beecham's pills. Gastro-intestinal cancer.	Beecham's pills. Non-gastro-intestinal disease.
Men	Under 45	11·8	4·2	2·9	3·5	5·9	3·5
	45–	7·2	3·1	4·3	1·6	4·3	4·2
	55–	9·2	4·4	7·1	1·5	7·1	4·2
	65+	8·3	3·9	2·5	2·5	10·0	4·4
Women	Under 45	6·9	10·0	10·3	5·0	10·3	7·5
	45–	17·2	7·3	6·9	7·3	10·3	7·3
	55–	20·5	9·3	11·0	9·3	17·8	14·0
	65+	11·1	5·3	6·7	5·3	12·2	5·3

striking, and, except for females under 45 years, the age specific rates for gastro-intestinal cancer patients were at least double those for non-gastro-intestinal patients. The differences in the senna rates—while showing the same trend—were not so marked or consistent, and though the age-specific rates of Beecham's pills' usage were without exception higher amongst gastro-intestinal cancer patients, the individual differences were, on the whole, small.

Direct comparison between the gastro-intestinal cancer and non-gastro-intestinal groups was carried out for each of the three purgatives, making appropriate adjustment as before for the differing age and sex structures of the two populations (Table VI). The most striking difference between the two groups was

TABLE VI.—*Comparison of Use of Liquid Paraffin, Senna, and Beecham's Pills between* (a) *Patients with Gastro-intestinal Cancer, and* (b) *Patients with Non-gastro-intestinal Disease.*

Purgative.	Gastro-intestinal cancer. Never.	Occasionally.	Often.	Total.	Non-gastro-intestinal disease. Never.	Occasionally.	Often.	Total.	χ^2.
Liquid paraffin—									
All ages: Obs.	545	21	48	614	1255	23	35	1313	13·96
Exp.	564·6	15·6	33·8	614	1235·4	28·4	49·2	1313	$P<0·001$
Senna—									
All ages: Obs.	576	6	32	614	1279	12	22	1313	6·54
Exp.	584·2	6·8	23·0	614	1270·8	11·2	31·0	1313	$P=0·04$
Beecham's Pills—									
All ages: Obs.	554	18	42	614	1251	22	40	1313	5·44
Exp.	566·1	14·4	33·5	614	1238·9	25·6	48·5	1313	$P=0·07$

again found in their use of liquid paraffin. There was a marked excess of liquid paraffin medication amongst patients with gastro-intestinal cancer. This difference was highly significant ($\chi^2 = 13·96$, $n = 2$, $P < 0·001$) and remained so when patients with cancer of the large bowel and cancer of the stomach were

separately compared with non-gastro-intestinal patients (χ^2 11·25 and 10·84 respectively; $P < 0{\cdot}01$ in each case). The comparisons relating to the use of senna and Beecham's pills show similar, but less marked, trends towards heavier usage by the gastro-intestinal cancer patients. The excess of senna in the gastro-intestinal cancer group just reached the level of technical significance and while a similar picture was presented by the separate consideration of cancer of the large bowel ($\chi^2 = 7{\cdot}21$, $n = 2$, $P = 0{\cdot}03$),—there was no significant difference between the experience of patients with cancer of the stomach and those suffering from a non-gastro-intestinal complaint. The third comparison, relating to the usage of Beecham's pills, showed no significant excess amongst the gastro-intestinal patients when considered collectively, or on breakdown to cancer of the large bowel ($\chi^2 = 4{\cdot}36$, $n = 2$, $P > 0{\cdot}1$) and cancer of the stomach ($\chi^2 \times 3{\cdot}52$, $n = 2$, $P > 0{\cdot}1$). Thus after due allowance for the differing age and sex structures of the two populations, it would appear that only in the use of liquid paraffin does there remain any worthwhile evidence in support of the apparent excess being real. Examination of the ratios between observed and expected numbers in the three categories of liquid paraffin usage *i.e.*, "never," "occasionally" and "often," supports this evidence. The ratios are respectively 0·97, 1·35 and 1·42, and they thus display a biological gradient of increasing "excess" with heavier usage of liquid paraffin.

TABLE VII.—*Duration of Purgative Medication Amongst Patients "Often" Taking Liquid Paraffin.*

Duration in years.	Gastro-intestinal cancer.				Non-gastro-intestinal disease.			
	Males.	Females.	Total.		Males.	Females.	Total.	
			No.	%			No.	%
Under 10	3	3	6	13	8	1	9	26
10–19	5	5	10	21	2	4	6	18
20+	12	20	32	67	14	5	19	56
Not known	0	0	0	—	1	0	1	—
All durations	20	28	48		25	10	35	

Study of the duration of purgative medication amongst patients taking liquid paraffin "often" also suggested a similar pattern (Table VII). Comparison between gastro-intestinal cancer and non-gastro-intestinal groups showed a higher proportion of patients in the gastro-intestinal cancer group had been taking liquid paraffin for as many as 20 years, and conversely there was a higher proportion of non-gastro-intestinal patients whose purgative medication extended over a period of less than 10 years. This difference, however, may be due to the differing age and sex structure of the two groups; the relatively small numbers available did not permit any useful comparison within separate age and sex groupings.

DISCUSSION.

Liquid paraffin is defined by the 'British Pharmacopoeia' as "a mixture of liquid hydrocarbons obtained from petroleum." According to Wood and Osol (1943) it "is made by distilling the residuary liquid boiling between 330° C. and

390° C. obtained after removing the lighter hydrocarbons from petroleum. It is purified and decolorized by first treating it with sulfuric acid and then with caustic soda and passing it while hot through animal charcoal. By cooling some solid paraffins will separate; the liquid is then redistilled, and the portion boiling below 360° C., rejected." In view of the known carcinogenic activity of crude petroleum, much attention has been paid to the possibility that liquid paraffin may contain carcinogenic fractions. The process of purification is likely to remove the greater part, it not all, of the polycyclic compounds initially present, but on at least one occasion samples of liquid paraffin offered for human consumption contained fluorescent substances (Trevan, personal communication) and the extent to which the original oil is purified is likely to vary from time to time and from manufacturer to manufacturer.

Tests on animals have always proved negative (Twort and Ing, 1928; Wood 1930) and liquid paraffin has been used as a vehicle for testing the carcinogenicity of other substances. These observations do not, however, entirely exclude the possibility that it may play some part in human carcinogenesis, either as a very weak carcinogen or in an ancillary role as a co-carcinogen or an accelerator.

The present data suggest that liquid paraffin may play such a part in a small proportion of cases of gastro-intestinal cancer in man. They do not prove it has such an effect. The data were obtained incidentally in the course of an investigation which was primarily concerned with the study of another type of cancer and more detailed and more accurate information from a larger number of cases is necessary before much weight can be attached to them. Moreover even if the basic data are considered to be sufficiently reliable, they are open to the interpretation that the usage of liquid paraffin is itself correlated with some factor which predisposes to the development of cancer of the stomach and large bowel. In this respect it is necessary, for example, to test whether the use of liquid paraffin may not be commoner in one or other social class or be prescribed because of the development of some other lesion of the gastro-intestinal tract, which predisposes to the development of cancer.

Since the period 1905–1910, when the medicinal use of liquid paraffin was first introduced at all extensively, there has been no important increase in the mortality from cancer of the stomach and large bowel; in recent years the mortality has even slightly decreased. Furthermore, liquid paraffin is used more commonly by women, yet the mortality from cancer of the stomach and large bowel is greater in men (in 1952, the crude mortality rate was 17 per cent higher in men than in women). It is, therefore, unlikely that liquid paraffin could be an important cause of either condition. Such differences in mortality are, however, not necessarily in conflict with the results of the present study, since if the data are interpreted to mean that liquid paraffin does contribute to the carcinogenic process, they also imply that the proportion of cases in which it is concerned is of the order of only 5 per cent.

SUMMARY.

The purgative histories of 614 patients diagnosed as having a gastro-intestinal cancer were compared with similar histories of 1313 patients who were suffering from diseases other than gastro-intestinal. No significant difference was discovered between the groups as regards their overall use of purgatives. Study of

individual purgatives revealed an appreciable excess amongst gastro-intestinal cancer patients only in the use of liquid paraffin.

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EFFECT OF NITROGEN MUSTARD OR X- OR RADIUM-IRRADIATION TREATMENT ON THE LEVEL OF BLOOD SERUM PROTEIN-BOUND CARBOHYDRATE (POLYSACCHARIDE) IN PATIENTS WITH NEOPLASTIC DISEASES.

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MANY observations have been published on the effect of disease or injury on the concentration of protein-bound carbohydrate (polysaccharide, protein sugar) or various carbohydrate-rich protein fractions in the blood serum, both in human beings and in animals. A study of the level of serum polysaccharide in various diseases led Seibert, Seibert, Atno and Campbell (1947) to suggest that a raised concentration is associated with tissue destruction. However, Shetlar, Bryan, Foster, Shetlar and Everett (1949) put forward an apparently opposing view, namely, that such increases are a reflection of tissue proliferation or repair rather than of destruction.

This paper deals with an investigation undertaken to find out what effect nitrogen mustard (the bis compound, di(2-chloroethyl) methylamine hydrochloride, was used throughout) or irradiation treatment might have on the serum polysaccharide level in patients with various neoplastic and allied diseases. (In such patients raised values are usually found.) It was hoped that some light might be thrown on any relationship between the polysaccharide level and tissue proliferation or destruction, since the nitrogen mustards and X-irradiation are known to cause tissue destruction or at least to inhibit actively growing tissues. Another object was to find out how much of any increase was contributed by the mucoprotein fraction. A preliminary report of the main findings has been published (Keyser, 1952*a*).

METHODS.

Fourteen cases were studied, made up of Hodgkin's disease (7), cerebral tumour (1), carcinoma of various types (4), chronic myeloid leucaemia (1) and reticulum cell sarcoma (1).

Blood was obtained by venepuncture immediately before treatment began, then at intervals during and after the course, the serum being separated and kept frozen until the analyses were performed. Total serum protein was separated by alcoholic precipitation (Keyser, 1952*b*). Mucoprotein was separated as described by Winzler, Devor, Mehl and Smyth (1948). Carbohydrate estimations were made by the orcinol method (Tillmans and Philippi, 1929; Sørensen and Haugaard, 1933) as described by Rimington (1940). Normal values found in this laboratory by these methods are as follows (Keyser, 1952*b*), expressed as mg.

hexose per 100 ml. serum : total serum protein carbohydrate, 102 to 139 (mean 119, standard deviation 12) ; mucoprotein carbohydrate, 10·0 to 13·0 (mean 11·9, standard deviation 1·2). In a few cases the albumin polysaccharide was determined after fractionation of the serum proteins with 26 per cent sodium sulphate (Majoor, 1946 ; Milne, 1947 ; Kibrick and Blonstein, 1948).

Serum, 1 ml., was incubated at 37° C. with 20 ml. of 27·2 per cent (W/V) sodium sulphate, Na_2SO_4, for about 4 hr. The precipitated globulin was filtered off with a 9-cm. filter paper (Whatman No. 5), and the albumin precipitated from 2- to 3-ml. portions of the clear filtrate by the addition of 0·25 volume of approximately 20 per cent perchloric acid and spun down after 10 to 15 minutes. The supernatant fluid was decanted and the tube inverted and allowed to drain. The protein was washed with 2 ml. of 95 per cent (V/V) ethanol and again spun down and the tube drained as before. Finally the protein was dissolved in 0·5 ml. of 0·125 N sodium hydroxide and the hexose estimated by the orcinol method as previously described.

Another portion (4 or 5 ml.) of filtrate was taken for estimation of the albumin.

In one case glucosamine was estimated in the precipitated serum total protein and albumin (26 per cent Na_2SO_4 fractionation), by the method of Elson and Morgan (1933) as described by Rimington (1940). Care was taken always to have approximately the same ratio of protein to acid in the hydrolysis from one specimen to another : results should therefore be comparable, though there are known to be numerous sources of error.

The serum tryptophan-perchloric acid reaction (Cohen, 1944 ; Seibert, Pfaff and Seibert, 1948) was performed on many specimens of serum. In a few cases urine was collected in an attempt to assess the excretion of total nitrogen, creatine, uric acid and amino-nitrogen. Because of the absence of facilities for accurate metabolic work it is possible that not all the urine collections were accurately made ; nor was it practicable to carry out nitrogen balances. For these reasons the urine findings are not presented in detail.

RESULTS.

In general, treatment that resulted in marked clinical improvement and reduction in the size of the tumour or glands was associated with a fall in the level of serum polysaccharide (total and mucoprotein) towards normal values ; and with relapse the polysaccharide level again rose.

Sometimes, however, during a course of treatment there was a preliminary rise in the level of polysaccharide before it fell. (It may be that this preliminary rise occurs in all cases. To determine whether this is so it would be necessary to take blood samples at more frequent intervals than we did, but this is not always justifiable.) The tryptophan-perchloric acid reaction paralleled roughly the total serum polysaccharide. No consistent changes in any of the urinary nitrogenous constituents were found during nitrogen mustard treatment (Cases 1, 2, 3 and 14). Creatinuria occurred in Cases 1, 2 and 3 during nitrogen mustard treatment, but not in Case 14.

Table I summarizes our findings. Essential case notes are as follows :

Case 1.—W. P—, a man, aged 50, suffering from Hodgkin's disease, was admitted to hospital on 30.iii.50 for his second course of nitrogen mustard. Since the first course of treatment a few months previously there had been an increase in

TABLE I.—*Summary of Findings in Fourteen Cases.*

Case No.	Age.	Sex.	Diagnosis.	Special treatment (N.M. = nitrogen mustard).	Date.	Serum total protein (g./100 ml.).	Polysaccharide (as mg. galactose-mannose/100 ml.). Total protein.	Mucoprotein.	Remarks.
1.	50	M.	Hodgkin's disease	N.M. Six 5 - mg. injections 31.iii.50–5.iv.50	31.iii.50	6·5	180	—	Greatly improved as result of treatment, especially about 3–4 weeks after end of course.
					3.iv.50	6.15	160	—	
					5.iv.50	5·95	154	—	
					6.iv.50	6·25	148	—	
					6.v.50	7·4	142	—	
				N.M. Six 5 - mg. injections	11.vii.50	6·35	194	—	Ditto.
					19.viii.50	7·2	139	—	
					16.ix.50	7·05	153	—	
				N.M. Four 5-mg., one 6-mg. and one 7-mg. injection 9–14.xii·50	8.xii.50	6·05	188	42·5	Not such good improvement after this course.
					11.xii.50	6·0	194	50·0	
					15.xii.50	6·1	214	54·5	
					20.i.51	7·2	143	19·0	
					14.iv.51	6·45	258	64·0	14.iv.51: Patient appears very ill. Died 12.vii.51.
2.	36	F.	Hodgkin's disease	N.M. Six 5 - mg. injections 18.iv.50–23.iv.50	18.iv.50	6·9	231	—	
					20.iv.50	6·7	235	—	
					25.iv.50	6·15	230	—	Discharged 27.iv.50 for convalescence.
					11.v.50	7·7	189	—	
					28.viii.50	7·5	253	—	Re-admitted 24.viii.50 (relapse).
3.	59	M.	Hodgkin's disease and pleural effusion	Aspiration of chest, 8.iv.50 and 14.iv.50	19.iv.50	5·35	160	—	
					20.iv.50	5·6	169	—	
					22.iv.50	5·6	170	—	
				N.M. Six 5 - mg. injections 20.iv.50–25.iv.50	25.iv.50	5·9	174	—	
					9.v.50	6·0	159	—	Discharged 17.v.50, oedema and chest signs reduced. Died 28.v.50.
4.	26	M.	Chronic myeloid leukaemia	N.M. Six 5 - mg. injections 30.vi.50–5.vii.50	30.vi.50	6·5	145	—	Improved on treatment.
					6.vii.50	7·15	151	—	
					10.viii.50	7·6	118	—	
					1.ix.50	7·65	144	—	Relapse.

Case No.	Age.	Sex.	Diagnosis.	Special treatment. (N.M. = nitrogen mustard.)	Date.	Serum total protein (g./100 ml.).	Polysaccharide (as mg. galactose-mannose/100 ml.) Total protein.	Polysaccharide (as mg. galactose-mannose/100 ml.) Muco-protein.	Remarks.
5.	76	M.	Reticulum cell sarcoma	N.M. Six 5 - mg. injections 10.ii.51–15.ii.51	6.ii.51	6·5	180	28·0	
					10.ii.51	6·55	198	—	
					14.ii.51	7·15	211	—	
					15.ii.51	7·0	204	31·0	
					19.ii.51	6·15	174	27·0	Discharged 23.ii.51.
					21.iii.51	6·85	240	46·0	Later admitted to hospital, and died 27.iii.51.
6.	58	M.	Hodgkin's disease	N.M. 5 mg. on 28.ii.51 and 1, 2, 5 and 7.iii.51	8.viii.50	5·9	180	—	
					28.ii.51	4·45	166	58·0	
					1.iii.51	4·25	174	—	
					3.iii.51	4·0	182	56·0	
					8.iii.51	4·0	186	58·0	
					10.iii.51	4·25	204	60·0	Died 14.iii.51.

Case No.	Age.	Sex.	Diagnosis.	Special treatment. (M.S.D. = maximum skin dose. G.D. = given dose.)	Date.	Serum total protein (g./100 ml.).	Polysaccharide (as mg. galactose-mannose/100 ml.) Total protein.	Polysaccharide (as mg. galactose-mannose/100 ml.) Muco-protein.	Remarks.
7.	71	M.	Squamous carcinoma of dorsum of left hand	Radium mould. Dose 5500 r. 79·4hr. in 8 days (21–28.ix.50). Estimated integral dose 8 M.gm. r. (very approximate, chiefly effect of the Ra on the whole body)	20.ix.50	7·05	148	25·0	
					28.ix.50	7·25	148	21·0	
					20.xi.50	—	155	22·5	20.xi.50: Healing.
					18.xii.50	7·15	146	24·5	18.xii.50: Healed. Covered with scar. Died 9.iv.51 (myocardial degeneration).
8.	39	F.	Carcinoma of left breast	Deep X-rays (palliative). 220 kv. H.V.L. = 1·5 mm. Cu. Dose: M.S.D. 3000 r. G.D. 3000 r. (each field). Time: 16 exposures in 10 days (20–29.ix.50). Estimated integral dose 7 M.gm. r.	20.ix.50	6·75	203	65·0	
					29.ix.50	6·6	239	64·0	
					29.xi.50	7·45	148	41·5	29.xi.50: Feeling better. Breast healing, but left axilla and left supraclavicular fossa may need treatment. Died: 22.iii.51.

TABLE I.—*Summary of Findings in Fourteen Cases—cont.*

Case No.	Age.	Sex.	Diagnosis.	Special treatment. (G.D. = given dose.)	Date.	Serum total (g./ protein 100 ml.).	Polysaccharide (as mg. galactose-mannose/100 ml. Total protein.	Mucoprotein.	Remarks.
9.	69	M.	Carcinoma of right upper alveolus involving cheek, palate, mouth	Deep X-rays (palliative). 220 kv. H.V.L. = 1·5 mm. Cu. G.D. 3296 r., right face. G.D. 2496 r. left face. Time: 16 exposures in 10 days (21–30.ix.50). Estimated integral dose 3 M.gm. r.	20.ix.50	6·55	212	37·0	Good response to treatment, but died 12.ii.51 (metastases in cervical glands).
					30.ix.50	7·0	181	30·0	
					15.xi.50	6·85	165	21·5	
					13.xii.50	—	—	43·5	
10.	45	M.	Carcinoma of skin of left forearm (? Pneumoconiosis.)	Radium mould. Dose 6000 r. Time 73 hr in 8 days (28.ix–5.x.50). Estimated integral dose 8 M.gm. r.	26.ix.50	7·3	149	14·0	13.x.50: Block dissection of glands in left axilla.
					6.x.50	7·45	148	—	
					20.xi.50	7·05	143	18·5	20.xi.50: Still has large area at primary site which is slow to heal.
					12.ii.51	7·15	123	11·0	15.i.51: Primary slowly healing.
					12.iii.51	7·65	138	18·0	12.iii.51: Small raised area at lower part of primary. No glands.
					16.vii.51	7·45	156	10·5	
11.	37	M.	Left frontal cerebral tumour	Deep X-rays (palliative). 250 kv. H.V.L. = 1·5 mm. Cu. Dose: T.D. 4000 r., G.D. 2700 r. (each field). Time: 45 exposures in 21 days (3–23.x.50). Estimated integral dose 4 M.gm. r.	26.ix.50	7·05	169	31·0	
					24.x.50	6·75	118	14·0	
					15.xi.50	6·75	147	19·5	
					7.iii.51	7·2	164	17·0	
					1.viii.51	7·25	150	13·0	
12.	30	F.	Hodgkin's disease	Deep X-rays (radical). 250 kv. H.V.L. = 1·5 mm. Cu. Dose: T.D. 3500 r. G.D. 2925 r. (right and left neck). Time: 30 exposures in 19 days (2–20.x.50). Estimated integral dose 9 M.gm. r.	27.ix.50	6·95	160	22·0	
					21.x.50	7·00	170	24·0	13.xi.50: Well. Neck glands regressing well. No fresh glands.
					13.xi.50	6·25	149	16·0	
					11.xii.50	7·0	124	9·5	
					7.iii.51	7·1	158	16·0	2.iv.51: Left tonsil still prominent.
					30.vi.51	6·95	198	22·5	
					3.xii.51	6·9	196	30·0	
					14.i.52	6·45	176	22·0	
					10.iii.52	6·8	184	28·0	
					21.iv.52	6·3	216	38·5	
					21.vi.52	6·0	218(61)*	26·5	21.vi.52:

							21.iv.52	6·3	216	38·5		
							21.vi.52	6·0	218(61)*	26·5	21.vi.52: Serum albumin 3·1	
					23.vi.52: 4 mg. N.M.		24.vi.52	6·0	228(61)*	31·5	24.vi.52: Serum albumin 3·2	
					24.vi.52: 4 „ „		26.vi.52	6·0	230(64)*	36·5	26.vi.52: Serum albumin 3·0	G./100 ml.
					25.vi.52: 5 „ „							
					26.vi.52: 6 „ „		28.vi.52	6·05	230(64)*	32·0	28.vi.52: Serum albumin 3·1 5.vii.52 Serum albumin 4·0	
					30.vi.52: 1 pint blood		5.vii.52	6·65	196(60)*	27·5	21.vii.52: Serum albumin 4·5	
							21.vii.52	6·45	152(46)*	19·5	21.vii.52: Looks well.	No glands
							22.ix.52	7·0	220(46)*	43·5	palpable.	
							2.ii.53	5·9	214(46)*	43·5	22.ix.52: Serum albumin 3·1 2.ii.53: Serum albumin 3·1	G./100 ml.
							* Figures in parentheses represent the polysaccharide in the albumin fraction				June 1953: Died.	
13.	49	F.	Hodgkin's disease	Deep X-rays. Radical: 250 kv. Dose: T.D. 3000 r., G.D. 3006 r. Anterior chest. Posterior chest. Time: 36 exposures in 21 days (2–23.x.50). Estimated integral dose 20 M. gm. r.			27.ix.50	6·45	247	78·0		
							24.x.50	6·3	155	29·0	23.x.50: Gland mass receding satisfactorily.	
							13.xi.50	6·7	136	12·0		
14.	—	M.	Hodgkin's disease	N.M. Six 5-mg. doses, 21–25.i.52			5.i.52	7·0	183	27·0		
							22.i.52	6·5	218	34·0		
							24.i.52	6·45	202	37.0		
							26.i.52	6·2	175	32·0		
							28.i.52	5·9	150	25·0		
							5.ii.52	7·0	142	22·0		
							16.ii.52	—	130	13·0		
							5.iv.52	6·5	122	19·0		
							1.vi.52	6·1	144	20·0		
							30.viii.52	6·5	168	21·5		
							7.iii.52	6·5	171	27·0		

the size of the glands. Daily injections resulted in improvement corresponding with a fall in the total white cell count and total polysaccharide, which was down nearly to normal a week after the beginning of treatment (see Table I). About the middle of June the patient began to complain of weakness and tiredness and noticed that the glands in his neck were once more increasing in size. He was re-admitted on 10.vii.50 and again nitrogen mustard produced clinical improvement and a fall in both white cell count and total polysaccharide. After another relapse he was given a fourth course of nitrogen mustard from 9.xii.50 to 14.xii.50. The patient was discharged, a little improved, on 18.xii.50. This time there was an increase in the polysaccharide (total and mucoprotein) during treatment but by a few weeks after the end of the injections it had again fallen nearly to normal levels. Unfortunately serial white cell counts were not made during the last period. The patient was last seen on 14.iv.51, when he appeared quite ill and his total and mucoprotein polysaccharides were very high. He died on 12.vii.51.

Case 2.—E.W—, a woman, aged 36, with Hodgkin's disease, was admitted to hospital on 18.iv.50 for a course of nitrogen mustard therapy. She had previously had four such courses. During treatment there was a slight rise in the serum total polysaccharide, then a fall. A leucocytosis apparently also occurred during the first few days but the white cell count fell markedly during the latter part of the course. Pyrexia persisted during treatment. No significant fall in the polysaccharide level occurred during the course of injections, but within 3 weeks after the end of the course it had fallen by 40 mg. per 100 ml. The patient was discharged to a convalescence home on 27.iv.50 and then home on 12.v.50. She felt very well until about the end of July, but she was again admitted in the following month with marked enlargement of the lymph glands of the neck and enlargement of the axillary glands. Her serum polysaccharide on admission was again very high. Unfortunately it proved exceedingly difficult to obtain further specimens of blood from this patient, on account of venous thrombosis, so that no further systematic study of the case was possible. She was later admitted to another hospital for radiotherapy and died there.

Case 3.—O.K—, a man, aged 59, with Hodgkin's disease and a left pleural effusion, was admitted on 4.iv.50 for nitrogen mustard treatment. After aspiration of the chest and six daily injections of nitrogen mustard the oedema of his arms improved considerably and "chest signs" improved. He was pyrexial during most of the course of treatment. On 17.v.50 he was discharged for convalescence, but he died eleven days later. There was a raised polysaccharide level on admission and no significant change during or after treatment.

Case 4.—A.W—, a man, aged 26, had chronic myeloid leucaemia. He was admitted to hospital for a course of nitrogen mustard and was subsequently discharged ("improved"). The serum polysaccharide fell after treatment, but was again slightly raised on readmission in September, when the patient was thin and pale and showed evidence of loss of weight. There were firm, mobile, moderately enlarged glands palpable in his neck, axillae and groins.

No further biochemical studies were made in this case.

Case 5.—G.R—, a man, aged 76, had noticed the appearance of large masses in the right side of his neck over the previous 18 months with a rapid increase in size in the 6 weeks preceding his admission to hospital. There were also lumps under his axillae and in the groins. A biopsy of a lymph gland on 3.ii.51 showed reticulum cell sarcoma. A course of six daily injections of nitrogen mustard

was followed by a slight reduction in the size of the lymph nodes and the patient was discharged ("improved") on 23.ii.51. During the nitrogen mustard treatment his serum polysaccharide (total and mucoprotein) rose somewhat, and it then fell but did not reach normal. The white cell count fell during treatment. Soon after discharge he was admitted to another hospital. His serum total and mucoprotein polysaccharide were found to be very high, and he died on 27.iii.51.

Case 6.—F.W—, aged 58, had previously received nitrogen mustard treatment for Hodgkin's disease, and on admission showed multiple skin nodules. He was given injections of nitrogen mustard. As shown in Table I the total serum polysaccharide increased steadily, but the mucoprotein polysaccharide showed only slight changes. The patient died on 14.iii.51. *Post mortem*, liver, spleen and mediastinal glands showed diffuse involvement.

Case 7.—T.C—, a man, aged 71, had had a wart on the back of his hand for several years. During the 8 months immediately before admission it had increased in size (on admission it measured 5 × 3 × 0·75 cm.) and for several weeks it had been ulcerating. A biopsy showed squamous carcinoma. The patient was treated with radium from 21.ix.50 to 28.ix.50. There were only slight changes in the polysaccharide, which was only slightly above normal on 20.xi.50, when the hand was stated to be healing. By February, 1951, it was completely healed, but the patient was taken ill about January and died on 9.iv.51 (myocardial degeneration).

Case 8.—V.C—, a woman, aged 39, had noticed a swelling on her left breast 20 years previously. It had been ulcerating for 4 years. On examination it was found that the whole of the left breast was involved by growth, and biopsy showed squamous carcinoma. There was no radiological evidence of secondary deposits in chest, thorax or pelvis but there was body destruction of the radicles and laminae of vertebra L3. At the end of deep X-ray therapy the total polysaccharide had increased by 36 mg. per 100 ml. but the mucoprotein fraction was about the same. At the end of 2 months, however, the total polysaccharide had fallen considerably and the mucoprotein fraction to a lesser extent. Healing of the medial half of the right breast area was progressing satisfactorily. The patient died a few months later. The cause of death was stated to be carcinoma of the left breast.

Case 9.—D.W—, a man, aged 69, had an extensive papillary squamous carcinoma which probably originated in the posterior part of a right upper alveolus but had spread medially to involve part of the soft palate and laterally to fill the cheek alveolus sulcus and was extending down on the posterior part of the right cheek. The size of the tumour was estimated at about 4 cm. in diameter in all axes. No glands were felt in the patient's neck. Biopsy of the palate revealed squamous carcinoma. The clinical response to treatment with deep X-rays was good. By the end of treatment the polysaccharide (total and mucoprotein) had fallen considerably and 6 weeks later it was still lower. On 13.xii.50, however, the mucoprotein polysaccharide was found to have increased sharply, and the patient died on 12.ii.51 (carcinoma of alveolus, metastases in cervical glands).

Case 10.—P.W—, a man, aged 45, had noticed a pimple developing on the dorsal side of the upper part of the forearm. On examination the growth was 23 sq. cm. in area. A section showed that it was a squamous cell neoplasm, probably malignant. Radium treatment was given from 28.ix.50 to 5.x.50, and

on 13.x.50 block dissection of glands in the left axilla was performed. No metastases, however, were seen in a section of the glands. Possibly the raised mucoprotein value on 20.xi.50 was a result of this operation. By 12.ii.51 the patient's polysaccharide (total and mucoprotein) was quite normal but it had increased slightly by 12.iii.51.

Case 11.—M.M—, a man, aged 37, had undergone partial removal of a left frontal oligodendroglioma in July, 1950. He had been subject to epileptic fits from March, 1947, but had had no fits or headaches since the operation. Deep X-ray treatment was given from 3.x.50 to 23.x.50. Both total and mucoprotein polysaccharide had fallen to normal levels by 24.x.50. Three weeks later, however, the total and mucoprotein polysaccharide were somewhat raised, and on 7.iii.51 the total had again increased somewhat but the mucoprotein polysaccharide had not. By 1.viii.51 the mucoprotein polysaccharide had fallen to normal levels and the total nearly to normal. To date, the patient has remained in good condition apart from occasional epileptic fits.

Case 12.—M.R—, a woman, aged 30, had had swelling of glands in the left side of her neck since December, 1948. In August, 1949, a mass was removed from under the left mandible and in April, 1950, a mass was removed from the posterior triangle on the left side of the neck. On examination (about September, 1950) the patient had a large mass of glands involving the whole left side of the neck, and the left tonsil was enlarged and fleshy-looking. Biopsy of the neck glands showed the presence of Hodgkin's disease. Deep X-ray therapy was given from 2.x.50 to 20.x.50. The white cell count fluctuated during, and the polysaccharide had increased by the end of, this period. On 13.xi.50 the patient was stated to be well, with the neck glands regressing satisfactorily and no fresh glands apparent. The left tonsil had also regressed. On 11.xii.50, by which time the polysaccharide level was normal, the glands were seen to have regressed and on 8.i.51 the neck was clear. An X-ray report (19.ii.51) stated that no secondary deposits were seen in the lung fields. The polysaccharide (total and mucoprotein) soon began to increase again, though the patient appeared to be in good health. After several months, however, she began to complain of tiredness and aching associated with a feeling of pyrexia in the evenings, for several days at a time. These symptoms were apparently unconnected with the menstrual cycle. A temperature chart kept by the patient from January to May, 1952, showed that on these days there was in fact a rise of temperature in the evenings, usually to 100–101° F. (Pel-Ebstein?). In June, 1952, the patient's condition had deteriorated and she was admitted to hospital for a course of nitrogen mustard injections. One pint of blood was also given (30.vi.52). The treatment produced a great clinical improvement and the serum polysaccharide level fell towards normal values. The distribution of polysaccharide between the serum protein fractions is shown in Table I. Normal figures found in this laboratory for the distribution of polysaccharide between these fractions are given in Table II.

The polysaccharide soon began to increase again and the patient's condition deteriorated. A further course of nitrogen mustard injections and transfusions of red blood cells in December, 1952, produced temporary improvement, but the patient's condition had again deteriorated by early February, 1953. Further transfusions in March again resulted in temporary improvement but the patient died in June, 1953.

Case 13.—A.B—, a woman aged 49, was found to have a mobile gland in the

TABLE II.—*Distribution of Protein-bound Carbohydrate between the Serum Albumin, Globulin and Mucoprotein Fractions in Four Normal Adults.*

Sex.	Total polysaccharide.*	Albumin polysaccharide.*	Globulin polysaccharide.*	Mucoprotein polysaccharide.*
1. M.	133	25	108	—
2. M.	114	21	70	14
3. M.	104	22	60	14
4. F.	130	23	93†	13·5

* As mg. hexose per 100 ml. serum.
† Calculated by difference.

left supraclavicular region and another in the left anterior axilla. X-ray examination (3.v.50) showed an opacity in the left upper zone, and a raised left diaphragm. Biopsy of the left supra-clavicular gland showed Hodgkin's disease with a good deal of necrosis and fibrosis. Nitrogen mustard was given from 13.vii.50 to 17.vii.50, before the case came to my notice. On 14.viii.50, glands were still present in the right and left supraclavicular regions, and by 11.ix.50 the right supraclavicular and left axillary glands were larger. X-ray of the chest now (11.ix.50) showed a large mass in the right superior mediastinum. Deep X-ray therapy was given from 2.x.50 to 23.x.50, by which date X-ray examination showed that the gland mass was receding satisfactorily. The polysaccharide (total and mucoprotein) had slowly fallen and was normal by 13.xi.50. By this time the mediastinal mass and glands had regressed, but X-ray examination suggested a new growth in the lung. On 11.xii.50 the glands were described as receding and the patient was feeling much better. Further X-ray examinations in January and March, 1951, showed a big opacity increasing in size, but no more specimens of blood were taken from this patient as venepuncture distressed her.

Case 14.—E.Y—, an adult suffering from Hodgkin's disease, was admitted in January, 1952, for a course of nitrogen mustard. (He had previously received nitrogen mustard in March, 1949, and September, 1950.) The sharp fall in the level of serum polysaccharide during treatment from 218 to 150 mg. per 100 ml. in the short space of 6 days is noteworthy. Glucosamine estimations suggested that most of the increase in polysaccharide from 5.i.52 to 22.i.52 and decrease from 22.i.52 to 5.ii.52 occurred in the albumin fraction.

DISCUSSION.

The view that a rise in serum polysaccharide results from tissue break-down is supported by the finding that increases occur in such diseases as cancer, tuberculosis and pneumonia, whereas in certain other diseases showing no tissue destruction comparable increases in the polysaccharide levels do not occur (Seibert *et al.*, 1947).

A similar interpretation may be applied to the rises in the α-globulin fraction of the plasma proteins that are found in febrile diseases (Longsworth, Shedlovsky and MacInnes, 1939) and after burning (Perlmann, Glenn and Kaufmann, 1943; Chanutin and Gjessing, 1946). The α-globulins are particularly rich in carbohydrate, and Seibert *et al.* (1947) found a correlation between the levels of serum α-globulin and polysaccharide in their series of cases.

Using the tryptophan-perchloric acid test (Cohen, 1944; Seibert *et al.*, 1948) we obtained evidence of a rise in the level of a serum constituent, that was believed

to be probably polysaccharide in nature, after burning (Keyser, 1949, 1950). The reaction has since been shown to be due to a polyhydroxylic acid of unknown structure (" sialic acid ") occurring in the proteins (Werner and Odin, 1952). More recently we have employed the orcinol method : both in burns and in fracture cases pronounced rises in the level of serum polysaccharide (total and mucoprotein) were found (Keyser, 1952*b*). This is consistent with the tissue destruction hypothesis, but the increase might equally well have been connected with healing processes.

That increases in the serum polysaccharide concentration occur in animals after experimental injury was shown by Shetlar, Bryan *et al.* (1949). The response was a delayed one, with a maximal elevation in 3 to 6 days after the injury. Increases in polysaccharide were produced by (among other methods) injection of talc suspensions or intrapleural injection of turpentine, causing conditions in which little tissue destructions was thought to occur ; fever was not essential for the elevation ; and the length of time that elapsed before the polysaccharide level returned to normal seemed to be correlated with the extent of repair taking place. The authors consequently considered it unlikely that the phenomenon was connected with destruction and suggested that it was connected in some way with tissue proliferation and repair. They do not, however, appear to have considered the possibility that a delayed breaking down of tissues as a result of the injury (" catabolic response ") may have occurred.

A full discussion of the evidence bearing on the various hypotheses will not be attempted here. According to Shetlar (1952) the available evidence indicates that the polysaccharide content of the albumin fraction is elevated in all cases in which tissue proliferation occurs ; that when a process involves fever the polysaccharide content of the α-globulin fraction increases ; and that if both fever (or perhaps tissue destruction) and tissue proliferation occur together the polysaccharide content of both α-globulin and albumin increases.

Our own results, described in this paper, might appear to favour the view that a rise in serum total and mucoprotein polysaccharide is connected in some way with tissue proliferation. In the cases studied by us it was noted that, generally, the best clinical response to treatment was associated with the sharpest fall in polysaccharide, which was, however, sometimes preceded by a slight rise.

(Shetlar, Foster, Kelly, Shetlar, Bryan and Everett (1949) mention rises in the serum polysaccharide level in cases of malignancy after X-ray therapy. They give no details, however, except in one case in which a small temporary rise is attributed to inflammation. This patient had previously undergone surgical removal of the carcinoma. Increases in the level of blood " polypeptides " as a result of radium and X-ray treatment were reported by Cristol and Puech in 1930. They did not extend their observations beyond the period of treatment).

As against this view it could be objected that the fall in polysaccharide seen after treatment was a secondary effect due to *inhibition of a breakdown process* in and around the tumour and depending on tumour growth. Such a break-down process might be expected to be greatest when the tumour is growing rapidly, and to be retarded when the tumour is subjected to an inhibitory influence such as that of the nitrogen mustards or irradiation. The fact that among the irradiated cases the most pronounced fall in the serum polysaccharide level was seen in the person receiving the greatest amount of irradiation (Case 13) appears to be consistent with either of these views. In this connexion the work of Catchpole and

his collaborators is of the greatest interest. Working with mice, Catchpole (1950) found that connective tissue bordering on tumours contained increased amounts of water-soluble mucoproteins; and histochemical studies suggest that in the region of tumours a process of depolymerization is taking place, whereby the components of the ground substance may become water-soluble and enter the general circulation as mucoproteins (Catchpole, 1950; Gersh and Catchpole, 1949). The existence of such a mechanism would go far towards reconciling the "tissue destruction" and "tissue proliferation" hypotheses, since the two processes—growth of the tumour and consequent depolymerization—would be associated with one another. It remains to be determined what bearing the depolymerization hypothesis has on the rises in the level of serum mucoprotein observed after injury. This and other aspects of the problem are now being studied.

In four of our cases urinary total nitrogen and nitrogenous constituents were measured in the hope that they might provide an indication of any tissue destruction taking place. That no consistent changes were found was perhaps to be expected, in view of the results of Danowski *et al.* (1950), who obtained no evidence of significant or consistent alteration in the electrolyte or nitrogen balances of patients under treatment with nitrogen mustard (di(2-chloroethyl) methylamine). Those authors point out that substances released by the breaking down of cells are disposed of by the anabolic, storage or excretory mechanisms of the body; and that, unless the first and second of these routes prove inadequate to deal with these substances, external balance studies will fail to show negative balances. In the experiments of Philips *et al.* (1948), who found variable but often extensive losses of nitrogen (and of sodium, potassium and chloride) after the injection of substances of this group into dogs, much larger doses per kilogram were used, and most of the animals died.

From the clinical point of view our results, especially in Cases 9 and 12, are of some interest. Although the number of cases studied is very small, the results do suggest that a further persistent increase in the polysaccharide level after it had been brought down by treatment denotes an unfavourable prognosis. It is also clear from the findings in Case 12 that a patient with a considerably raised serum polysaccharide level may appear to be in good health for some months before symptoms appear. These findings suggest that a knowledge of the level of serum polysaccharide might be of some prognostic value in patients receiving nitrogen mustard or irradiation treatment.

The rapidity with which changes in the polysaccharide level in the blood can occur is noteworthy (*cf.* Case 14). This might have an important bearing on the whole question of the metabolism and significance of the glycoproteins and mucoproteins.

SUMMARY.

In patients treated with di(2-chloroethyl) methylamine hydrochloride, or by radium or X-irradiation, a favourable clinical response was generally associated with a fall in the blood serum polysaccharide (total and mucoprotein) towards normal levels. During treatment there was often a preliminary rise. With relapse the polysaccharide again became elevated. The significance of these findings is discussed.

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MOLLUSCA PSEUDOCARCINOMATOSA. DISCUSSION OF A CASE OF THE FERGUSON SMITH TYPE OF UNILATERAL DISTRIBUTION.

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THERE are apparently three clinical types of spontaneously healing skin tumour [molluscum pseudocarcinomatosum (Editorial, 1953)] in which the diagnosis of squamous carcinoma, Grade 1, may be made microscopically; (*a*) the solitary type [molluscum sebaceum (MacCormac and Scarff, 1936), kerato-acanthoma (Rook and Whimster, 1950), or diverticule épidermique à paroi végétante (Dupont, 1952)]; (*b*) the multiple form, corresponding to Smith's (1934) epithelioma; and (*c*) a third in which hundreds of tumours may be present (Grzybowski, 1950; Witten and Zak, 1952).

The solitary type is now recognized as relatively common (Beare (1953) suggests that as many as a third of the lesions diagnosed as squamous carcinoma of the skin are mollusca pseudocarcinomatosa) and cases have been reported from many parts of the world. The Smith type is still, however, a relative rarity and less than a score of cases have been reported since the original description in 1934.

The following case, the second to be seen in South Africa, closely resembles that originally described by Smith (1934); but it is unique in that the tumours and scars are confined to the left side of the head and body.

Case History.

Mr. P. S—, a South African-born European of remote German and Dutch ancestry, aged 37, was first seen in December, 1953. He had suffered since 1942 from recurrent spontaneously healing tumours of the left face, ear, neck, chest, shoulder and upper arm. No other member of his family was known to have suffered from any similar disease; and he was not related to the other South African who suffers from the Smith type of mollusca pseudocarcinomatosa. His skin generally was normal for his age and was not hypersensitive to sunlight. His occupation as a contractor kept him mainly in the open. He had never been exposed to tars, mineral oils or arsenic.

He stated that the tumours, of which he usually had several active, began "like festering blackheads", grew for 4–6 weeks, and then began to regress and disappeared, leaving scars, in 4–6 months. On his first visit he had two active tumours on the back of the left ear and one over the left scapula and thirty depressed white scars on the left side of the nose, upper lip, cheek, ear, neck, chest and upper arm. The scalp and buccal mucosa were unaffected. No enlarged lymph glands were discovered.

The lesions on the ear were little crateriform tumours, 3 mm. and 5 mm., with hard, white, elevated rolled edges and a keratinous central mass. The lesion over the scapula was an elevated dome-shaped tumour, 2·5 cm., with a central infected keratinous mass. A month later the ear lesions were distinctly smaller and flatter and that over the scapula had flattened down to a coin-like plaque with rolled edges and a central area of soft keratinous material. All the lesions were clinically indistinguishable from squamous epitheliomata. At this visit a fresh lesion, 1 mm., was discovered in the angle of the left ear lobe and cheek; it closely resembled, as the patient had described, a festering blackhead.

The patient, who believed himself to be suffering from skin cancers, has on several occasions been subjected to biopsy or excision of tumours; and a diagnosis of squamous epithlioma had generally been made. X-ray therapy had also been given more than once with little impression on the rate of healing of the tumours; but the scars left in such areas were more mutilating than those seen after spontaneous healing.

A portion of one of the lesions on the ear was excised for histological examination and we examined several sections of lesions excised in the past by Dr. D. H. Thompson and prepared by Dr. J. Tulloch of Bulawayo. The features of the pseudo-epitheliomatous tumours are amply illustrated in the photomicrographs and, in view of the numerous descriptions in recent articles, no details are considered necessary.

DISCUSSION.

It is now generally agreed that the solitary and multiple varieties of mollusca pseudocarcinomatosa are clinical variants of a single entity; but the nature and cause of these tumours are still unknown.

It has been suggested that exposure to sunlight (Sommerville and Milne, 1950) is a factor in the appearance of the Smith type. Against this are the facts that solar sensitivity is not described as occurring in any case, that the tumours arise on apparently normal skin and that the lesions have appeared in several cases as frequently on covered as on uncovered skin. If sunlight were a factor we should expect the condition to be, like other solar dermatoses, much commoner in South Africa than in Britain.

In one spontaneously healing pseudocarcinomatous condition, Poth's tumour-like keratoses (Poth, 1939), sunlight always plays a major rôle in the appearance of tumours of exposed skin which closely resemble the lesions of molluscum pseudocarcinomatosum in their behaviour and histological appearance. It is not certain, however, whether sunlight is the only relevant factor in causing these tumours, because Poth's original case suffered from multiple flat warts (and one pathologist thought the histological picture suggestive of verruca vulgaris), and Fliegelman and Loveman's (1952) case had worked as a blacksmith and near tar (*cf.* Rook and Whimster's (1950) case with tar melanosis and kerato-acanthoma).

The theory of virus infection as a cause of mollusca pseudocarcinomatosa has some supporters, notably Fouracres and Whittick (1953). These authors would ascribe to infection the familial incidence of some cases of the Smith type. Inoculation experiments (Grzybowski, 1950; Beare, 1953; Marshall and Findlay 1953) have failed, so far, to confirm this theory; and the fact that the vast majority of cases are of the solitary type seems also to go against it.

Marshall and Findlay (1953) have suggested that the self-healing property of mollusca pseudocarcinomatosa may be due to their derivation from the hair apparatus and that the tumours are extruded after a time in the same manner as are hairs from the hair-follicle.

The unilateral distribution in this latest case raises yet another possibility—that the condition may be naevoid in origin. Such a distribution makes sun-sensitivity an unlikely cause and, to our minds, excludes an infective factor. Fouracres (1954, personal communication), however, reminds us that one virus infection, zoster, is almost always unilateral. Parkes Weber (1954, personal communication) takes our view and writes " I regard the infection theory as most extremely improbable, and would certainly classify the case under the heading of naevi of inborn origin or inborn predisposition—*cf.* my recent book ' On Naevi and Miscellaneous Subjects ' (Parkes Weber, 1952). Of course I mean naevi in the large sense of the term. The unilateral distribution of the lesions in your case strongly confirms the naevoid classification. Your case is in fact, to my mind, a variety of the various kinds of *naevus unius lateris* and analogous to unilateral freckling, etc., etc."

The problems of diagnosis and treatment are not great in cases with multiple tumours and scars of healed lesions ; but mistakes are bound to be made with the solitary tumours. The appearance and progress of the lesion may be suggestive and the friability of the tumour may be almost diagnostic (Beare, 1953), but world authorities on the pathology of tumours have reported such cases as squamous epithelioma, Grade 1.

We feel that, in the present state of our knowledge, complete excision of accessible solitary tumours, first seen in the growing stage, is the wisest policy.

SUMMARY.

A case of mollusca pseudocarcinomatosa of the Smith type is described. The case is unique in that the lesions, over a period of 11 years, were unilateral in distribution.

The nature of these tumours and their relationship to other self-healing pseudocarcinomatous lesions is discussed.

It is suggested that molluscum pseudocarcinomatosum may be of naevoid origin.

We are indebted to Dr. D. H. Thompson and Dr. J. Tulloch, of Bulawayo, for specimens of tumours excised and examined by them ; and Fig. 2 illustrates one such specimen.

The photomicrographs were made by Dr. F. A. Brandt of the South African Institute for Medical Research, Johnannesburg.

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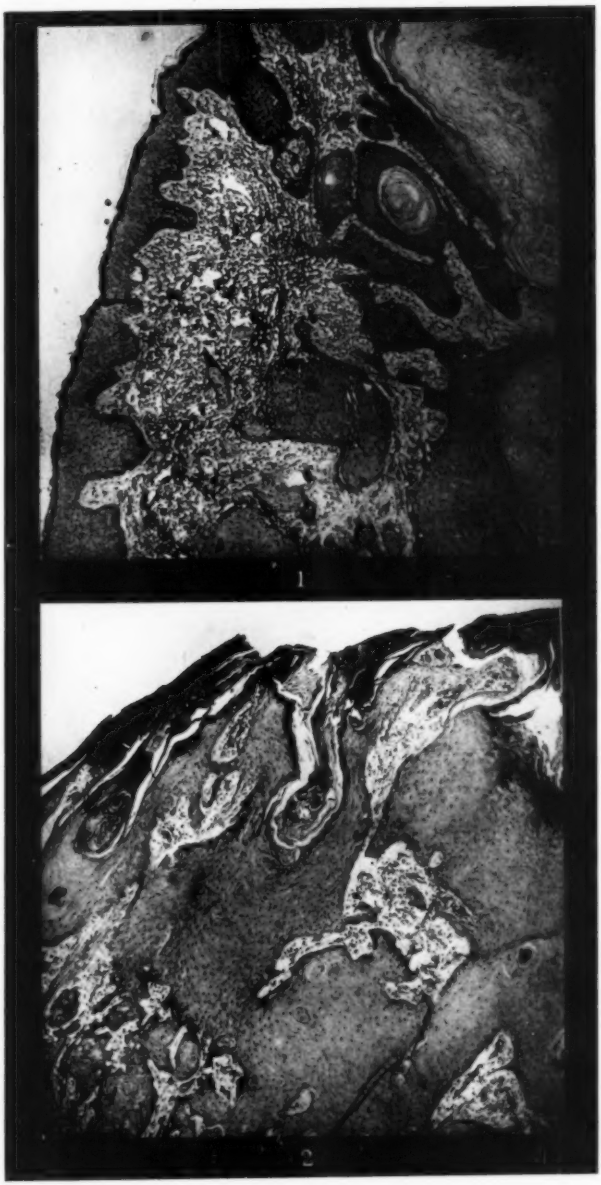
EXPLANATION OF PLATE.

Fig. 1.—Molluscum pseudocarcinomatosum (ear) showing central mass of keratin bounded mainly by atrophic epidermis. The stretched and atrophic epidermis covering the lesion is seen on the left. Between the central mass of keratin and the covering epithelium are irregular and well-differentiated masses of epithelium and cell nests. H. and E. × 60.

Fig. 2.—Molluscum pseudocarcinomatosum (neck) showing an early invasive lesion which cannot be differentiated from early squamous carcinoma on histological grounds alone. H. and E. × 60.



Marshall and Pepler.

THE EFFECT OF STILBOESTROL IN ACUTE LEUKAEMIA.

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THE following considerations led us to carry out a trial of stilboestrol in acute leukaemia. It is well known that the endocrine organs play an important part in the regulation of haemopoiesis, and many workers have reported the beneficial effects of ACTH and cortisone in some cases of this disease. Unfortunately, however, remissions so induced are usually only of short duration. Re-treatment may again have a beneficial effect, but eventually a refractory state is reached.

On the other hand, " myelokentric " and " lymphokentric " acids, steroid-like principles extracted from urine (Miller and Turner, 1943) and from serum (Foster and Miller, 1950) cause leukaemoid effects of myeloid and of lymphatic type respectively when injected into normal animals. Furthermore, myelokentric acid has induced partial remissions in human cases of lymphoblastic leukaemia in the hands of Miller, Herbutt and Jones (1947). Hence hormonal imbalance may play a part in the aetiology of leukaemia.

Finkelstein, Gordon and Charipper (1944) demonstrated that oestrogens have a depressant effect on the bone-marrow in certain conditions, but they did not study leukaemic states.

Burrows and Horning (1952) have shown that not only are oestrogens carcinogenic, but they may be carcinostatic, often restraining and causing regression of some human neoplasms such as carcinomata of the breast and prostate. They also state that the liability of mice to leucosis can be increased by oestrogens and reduced by androgens. Furth (1952), however, reminds us that in most strains of mice leukaemia is more common in the female, but in man it is more common in males. It is possible, therefore, that in human leukaemia oestrogens may have the opposite effect to that in mice.

Dausset and Schwarzman (1951) reported a study of 212 cases of acute leukaemia with regard to the influence of age and sex upon the frequency and cytological types. They also reviewed other series in the literature from these points of view, including both acute and chronic leukaemia. These authors state that between 18 and 50 years in both males and females there is an overwhelming preponderance of myeloid leukaemia, while under the age of 18 and over the age of 50 lymphoid leukaemia occurs almost exclusively. As the former occurs most commonly during the period of maximal procreative activity, they suggest that there may be an endocrine effect on the lymphatic-myeloid equilibrium, especially with regard to the sex hormones.

Stilboestrol appears to have had a beneficial effect in single cases of chronic myeloid leukaemia (Loeper, LeSourd and Sterboul, 1947) and of chronic lymphatic leukaemia (Lemaire, Loeper, Housset and Koupernik, 1947). On the other hand

Laroche, Tremolieres, Dausset and Oury (1949) report a favourable effect with high doses of testosterone in one case of acute myeloblastic leukaemia.

METHOD.

As the course of acute leukaemia, including the frequency of partial and complete remissions, is well known (Bierman, Cohen, McClelland and Shimkin, 1950; Rodgers, Donohue and Snelling, 1951; Southam, Craver, Dargeon and Burchenal, 1951), and any important modification as a result of treatment should therefore become evident, the cases were not divided into treated patients and untreated controls. Fifteen consecutive cases of acute leukaemia (8 with the myeloblastic and 7 with the monoblastic form) were treated with stilboestrol. This was given orally, the dosage in general being 5 mg. *t.d.s.* increasing by 5 mg total per day to 20 mg. *t.d.s.* Transfusion of packed red cells, antibiotic therapy, oral hygiene and simple psychological treatment were adopted as the respective clinical indications arose. Exchange transfusions and other remedies such as X-rays, radiomimetic drugs, ACTH and cortisone, liver extracts, folic acid and vitamin B_{12} were not used.

Diagnosis in each case was made by clinical and peripheral blood examinations and confirmed by bone-marrow studies.

RESULTS.

These are shown in Table I. The group of 15 patients consisted of 11 males and 4 females, their ages varying between $2\frac{1}{2}$ and 67 years. The average duration of symptoms prior to therapy was approximately 9 weeks, and after the commencement of treatment 48 days. No cases survived for more than 5 months after therapy was begun, and none showed a complete remission.

Only 3 cases showed partial remissions as judged by objective signs of clinical improvement (Cases 2, 6 and 10).

TABLE I.—*Cases of Leukaemia Treated with Stilboestral.*

Case No.	Type of leukaemia.	Age in years.	Sex.	Duration of symptoms before therapy in weeks.	Survival after commencement of therapy in days.	Total duration in weeks.	Total amount of stilboestrol given, in mg.
1	Myeloblastic	46	F.	2	2	2	40
2	,,	19	M.	6	152	28	4665
3	,,	20	M.	5	91	18	12
4	,,	49	F.	26	8	27	200
5	,,	21	M.	3	27	7	725
6	,,	20	F.	17	153	39	4462
7	,,	20	M.	7	9	8	280
8	,,	$2\frac{1}{2}$	M.	80	54	16	287
9	Monoblastic	67	M.	17	4	18	140
10	,,	53	M.	5	35	10	280
11	,,	44	M.	5	6	6	120
12	,,	37	M.	5	6	6	105
13	,,	27	M.	13	15	15	225
14	,,	15	M.	6	131	25	4442
15	,,	44	F.	7	21	10	60

Bearing in mind that remissions in acute leukaemia may rarely be spontaneous, or that they may be induced by blood transfusions and/or antibiotics, in 2 of

these 3 cases there was some suggestive evidence of transient benefit from stilboestrol itself. Case 2 had had penicillin therapy and three blood transfusions, each of two bottles of blood, over a period of 20 days elsewhere without improvement. His clinical condition had deteriorated, while the haemoglobin level had fallen from 48 per cent to 42 per cent, and the red cell count from 2·6 m. to 1·9 m. per c.mm. during this time. On admission to hospital in Sheffield he was given 3 pints of packed cells and stilboestrol and penicillin therapy were begun. This was followed by an excellent clinical remission for 6 weeks, with complete disappearance of clinical splenomegaly and hepatomegaly, the latter organs having been palpable 2 in. below the respective costal margins at the commencement of treatment. Two days after the blood transfusion the haemoglobin level was 8·9 g. per 100 ml. and the total leucocyte count 800 per c.mm. Over the next few weeks his haemoglobin level rose to 12·6 g./100 ml., while the total leucocyte count reached 6000 per c.mm., the only abnormal cell in the peripheral blood being an occasional (1 per cent) blast form. When Case 6 was admitted to hospital, she had gross gum hypertrophy, and a spleen palpable 1½ in. below the left costal margin. Her haemoglobin level was 7·8 g./100 ml. Ten days after the commencement of stilboestrol therapy only, she was subjectively much improved, the gum hypertrophy had diminished considerably, and the spleen was impalpable, while the haemoglobin level had risen to 8·1 g./100 ml. Three days later additional blood transfusion therapy was begun.

Stilboestrol was well tolerated in most cases, and toxic effects were few. Nausea and vomiting led to the abandonment of this therapy in Case 13 after 5 days, while Cases 6 and 15 experienced transient nausea only in the first few days of treatment. Slight mammary hypertrophy and increased areolar pigmentation were noted in Cases 2, 3, 6 and 14.

SUMMARY AND CONCLUSIONS.

Fifteen cases of acute leukaemia (8 myeloblastic and 7 monoblastic) were treated with stilboestrol. None lived for more than 5 months after beginning therapy. No complete remissions occurred, but 3 patients had transient partial remissions.

There is no evidence from this series to suggest that either the prognosis or the clinical course of acute leukaemia is altered by adding stilboestrol to other therapy.

I am indebted to my medical and surgical colleagues in Sheffield, to Dr. R. T. Gaunt, of Chesterfield, and to Dr. Peter Milligan, of Doncaster, for referring their cases. Thanks are also due to Professor E. J. Wayne and Dr. G. M. Wilson for reading and criticising the manuscript.

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A PHYSICAL APPROACH TO THE PROBLEM OF CANCER.

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THE changes which occur in the molecular structure of cells, when a tissue changes from a benign to a malignant form, appear to be of such a kind that they cannot be analysed by present physical or chemical techniques. While this may be true for the problem on a molecular scale, it does not follow, *ipso facto*, that an investigation cannot be made at a higher level of organisation. Hinshelwood (1952) has pointed out, for example, that the three-body problem in mechanics cannot be solved, although the kinetic theory can explain the behaviour of a large number of bodies. He has shown that bacterial growth can be understood in terms of certain simple mathematical relationships, in spite of the fact that the molecular mechanisms involved are extraordinarily complicated.

In this paper an attempt is made to see if such an approach may help to throw a little light on the problem of tumour formation and growth. A brief review is first given of the various physical, chemical and biological methods of producing tumours. This is followed by a mathematical analysis of the problems of growth and differentiation (Sections II and III); an attempt is then made to apply the methods to tumour formation and finally some experimental evidence is given to support the general conclusions.

I. *A Brief Review of Carcinogenesis.*

The various ways of producing cancer have been reviewed in a recent paper by Boyland (1952). But it will help for the purposes of the present communication to provide a summary of the results.

(a) *Chemical carcinogens.*—The first chemical compound to be identified as a carcinogenic agent was 1:2:5:6-dibenzanthracene (Cook, Hieger, Kennaway and Mayneord, 1932). Since that time, the polycyclic hydrocarbons have been investigated systematically from this point of view. A certain degree of specificity has been observed and it has been correlated to some extent with the presence of a region of high electron density (*K* region, Schmidt, 1939), within the molecule. Some recent work has suggested, however, that the carcinogenic activity of the polycyclic hydrocarbons may be related in part to their ability to undergo auto-oxidation and liberate free radicals (Alexander, 1952).

The second class of carcinogenic compounds includes mustard gas (Heston, 1950), nitrogen mustards (Boyland and Horning, 1949), epoxides, mesyl glycols (Haddow and Timmis, 1951), etc. These compounds are all known to act as alkylating or esterifying agents and probably produce their effects on the body by this type of reaction.

Certain inorganic salts of beryllium and zinc can produce tumours (Bagg, 1936). There are also a number of other chemical compounds, showing little relationship

to those mentioned above, which can induce the formation of tumours. Urethane (Nettleship and Henshaw, 1943) is an example of this kind. It might, from the nature of its amide group be expected to attack the secondary forces of cohesion known as hydrogen bonds, which play a most important part in maintaining the structure of biological organelles such as chromosomes (Ambrose and Gopal-Ayengar, 1952).

(b) *Physical methods of producing cancer.*—It appears that the simple process of implanting an inert object such as a disc of bakelite (Turner, 1941) or sheets of cellophane (Oppenheimer, Oppenheimer and Stout, 1948) into a tissue can produce a tumour, while an extreme change of temperature produced by the application of carbon dioxide snow (Berenblum, 1929) can have a similar effect.

Although the ionising radiations can induce cancer, it is probable that their mode of action is indirect and is due to the formation of free radicals (Butler and Conway, 1952).

(c) *Biological carcinogens.*—The well-known Rous sarcoma can be propagated by virus particles (Rous, 1936). Certain parasitic organisms are able to cause a malignant change to occur in the tissue (e.g., Phytomonas tumefaciens in plants, Smith, 1916). The spontaneous tumours, whose origins are unknown should perhaps be considered to be due to the action of a biological carcinogen.

It was pointed out by Haddow in 1938 that there was a striking multiplicity of tumour-producing agents and, as can be seen from the summary given above, the number has greatly increased since that time (Hartwell, 1951). The existence of so many agents suggests, at first sight, that we are dealing with a problem of baffling complexity. But there is just a possibility that the great variety may itself provide a clue as to the mechanisms involved. The agents appear to have one feature in common ; they may be expected to lead to a more random configuration of the molecular structure within the cell, than is present in the normal tissue. We will consider the effects which a change of this kind might be expected to produce upon the economy and energy requirements of the cell.

II. *A Mathematical Approach to the Problems of Growth and Differentiation.*

We will first attempt to consider problems of growth and differentiation in terms of certain simple models, to which the laws of thermodynamics can be applied. We will try, at a later stage, to see if the models may have any bearing on the behaviour of real biological systems.

(a) *Classical treatment.*

(1) *Simple growth.*—We will consider the very simple system shown in Fig. 1. The sphere of radius r_1 is considered to contain a dilute solution of metabolite molecules ; the molecules could, for example, be molecules of amino acids. It will be assumed that the solution is ideal ; i.e., that the molecules are quite independent of each other and do not interact with the solvent molecules. They behave like the molecules of an inert gas. Suppose that the molecules are surrounded by a hypothetical semi-permeable membrane, which allows free passage for the solvent but not for the metabolite molecules. If the metabolite molecules are compressed reversibly into the volume of the sphere of radius r_2 at constant temperature, an amount of work equal to $nRT \log V_1/V_2$ must be done for a fraction n of metabolite

molecules, where n is the molar fraction of molecules of this species. If there are other species present, these will require corresponding amounts of energy and the total will be the sum of all components $\Sigma n,RT \log V_1/V_2$.

Because no process can require less energy than a reversible process it follows that it is necessary to expend energy in this way in all cases of growth. The work which has to be done is generally known as osmotic work. In thermodynamic nomenclature the work which must be done on the system is in this case expressed as a change of entropy. The entropy of any system can be expressed as a measure of the degree of order or disorder which may be present, *a low entropy* corresponding to a *high state of order*. In the case described above, the growth process has involved a decrease of entropy.

Other thermodynamic concepts such as free energy changes are commonly used in studying chemical equilibria, but in this case we shall be concerned with the shape or microscopic morphology of biological material and it will be found that the entropy concept is more useful.

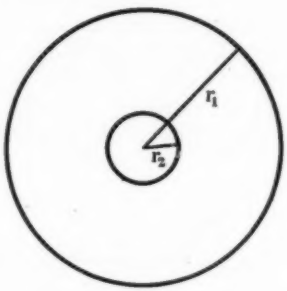

FIG. 1.

In real systems the energy required for the decrease of entropy is in some cases balanced by a change in the potential energy, e.g., the formation of a chemical precipitate is accompanied by a decrease of potential energy, because forces of attraction occur between the molecules forming the precipitate and a closer approach of the molecules leads to a decrease of potential energy.

(2) *Growth with differentiation.*—We will at this stage consider the problem of growth with differentiation since it has been the subject of a beautiful study by Tyler (1939), using sea urchin eggs, although the treatment is not in this case strictly thermodynamic.

The cells at the two cell stage of embryonic development are isolated. They will each continue to develop but they will not develop as the normal embryos. They contain half as many cells as the normal embryo at corresponding stages of development.

In Fig. 2*a* is shown a normal embryo at the blastomere stage of development. The external radius is R units and the wall thickness is d units. There is a single layer of cells forming the wall thickness. In Fig. 2*c* is shown a dwarf embryo, in which it is found experimentally that the surface area is half that of the normal embryo. The radius is therefore $R/\sqrt{2}$ units. The wall thickness, which is the thickness of a single cell, is the same as that of the normal embryo (d units).

At this stage of development invagination begins to take place as shown in Fig. 2*d*.

It is possible to compare the energy requirements for growth in the case of the normal and dwarf embryos by making use of the principle of dimensional analysis (Gallileo, Rayleigh, Tolman, Buckingham). These principles tell us that there is a relationship between all physical quantities and the dimensions in which they are expressed, so that by making use of this principle, it is possible to calculate the magnitude of any quantity, for a case on a different scale of dimensions from those originally chosen.

Alternatively, we may say that in a universe which was constructed on a different scale, e.g., with its linear dimensions half those of the present universe, we should find that this 1:2 relationship would apply also to the other physical quantities, which can be expressed in terms of mass length and time.

In Fig. 2*b* is shown a hypothetical dwarf embryo in which all the dimensions have been reduced in the ratio of $1:1/\sqrt{2}$. The wall thickness is $D/\sqrt{2}$ units and

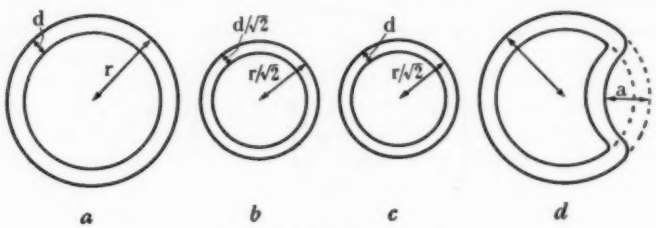

FIG. 2.
(In *d* the longer line between arrows indicates r.)

not d units as in the real dwarf embryo shown in Fig. 2*c*. Suppose that an invagination of the wall of the embryo takes place to the extent of a units in the normal embryo at gastrulation. Then an invagination of $a/\sqrt{2}$ units will take place in the hypothetical dwarf.

A process of deformation of the kind shown in Fig. 2*d* will require work, which is measured as a product of the applied force (F) and the distance moved (a), in the case of the normal embryo. There are two possible ways in which the deformation can take place. It can be either as a plastic or as an elastic deformation. Tyler (1939) considers that the deformation is plastic for a number of reasons, the main one being the fact that organisms do not tend to return to a spherical shape when the cells die.

For the plastic case, in which the work done is proportional to the square of the linear dimensions, the work is equal to $F.a$ units. In the case of the hypothetical dwarf, it will be, according to the methods of dimensional analysis, equal to $F/\sqrt{2}\times a/\sqrt{2} = F.a/2$.

In the case of the real dwarf the thickness of the wall is d and not $d/\sqrt{2}$. If the deformation is plastic the force required will be a linear function of the thickness and will be equal to $F/\sqrt{2}\times\frac{d}{d/\sqrt{2}} = F$. The work done will be $F.a/\sqrt{2}$. The two dwarfs together will require an amount of work equal to $2\times F.a/\sqrt{2}$ to be done, i.e., $F.a\sqrt{2}$ or 1·41 times as much as the normal embryo. It can be shown mathematically that if the deformation is elastic the work done will vary as the

cube of the linear dimensions and the difference between the energy requirements of the normal and dwarf embryos would be correspondingly increased.

It is found by experiment that the normal and dwarf embryos respire at the same rate but that there is a retardation in the rate of development of the dwarf embryos as compared with the normal of between 30 and 40 per cent. This means that the dwarf embryos require 30–40 per cent more energy for the differentiation process. The agreement with the theoretical prediction is good and signifies that the deformations involved in the growth process are probably largely plastic. If the deformations were elastic a considerably greater difference between the energy requirements would be expected in the two cases.

We see from these results that differentiation is an energy absorbing process.

(b) *Statistical mechanical treatment.*

The treatment given above, which is due mainly to Tyler (1939), represents a study of differentiation in terms of the tissues of the organism involved. We will now attempt an analysis of the problem on a molecular scale with hypothetical models, making use of known data concerning the physics of long chain molecules. High polymers provide the basic structural elements of all living systems, so that growth and differentiation are intimately connected with changes in such systems. As a preliminary treatment, we will neglect all changes of internal energy which may be involved in any process of polymerization or alteration in the configuration of the molecules.

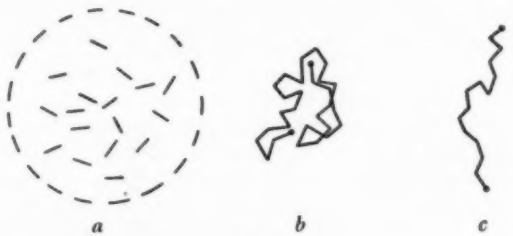

FIG. 3.

In Fig. 3*a* the metabolite molecules, of the kind which were described in Section II*a*, are represented as rod-like particles. They could for example be simplified molecules of amino acids. They are considered to be confined within the sphere of radius r_2 by a semi-permeable membrane, but they are not restrained in any other way. As before, they are considered to behave like the molecules of an inert gas. In Fig. 3*b* the molecules are shown to be arranged in a linear sequence, in the form of a polymer chain. In this case the chain is coiled in an irregular manner. In Fig. 3*c* the chain has been stretched, as it would be in the case of a piece of stretched rubber.

Let us consider the number of possible arrangements or conformations, as they are called, in the three figures. In Fig. 3*a* there are very few restrictions upon the arrangement of the metabolite molecules. It is only necessary that one molecule should not coincide with another and that they should all be confined within the sphere of radius r. In Fig. 3*b* we see that the number of possible ways of arranging the units is greatly reduced, because each unit must be maintained at a

certain distance from its immediate neighbours. The position of the ends x and y is not restricted however, in any way. It is possible to show that there is a most probable distance apart of the ends x and y, proportional to the square root of the number of units in the chain.

In Fig. 3*c* the restriction upon the position of the ends has produced a further decrease in the number of possible conformations. A statistical thermodynamic treatment makes it possible to express the entropy (S) of the systems in terms of the number of conformations with the most probable energy. If n is the number of conformations with this energy:

$$S = k \log n, \text{ where } k \text{ is a constant.}$$

The logarithmic form occurs because the result is obtained by integration over a large number of terms.

It will be seen that the simple process of polymerization must lead to a decrease in the entropy of the systems and that any process leading to a preferred orientation of the polymer molecules must lead to a further decrease.

Let us now consider a case in which we have a number of the spherical units of the kind shown in Fig. 3. In Fig. 4*a* each circle represents a group of long chain molecules surrounded by a semi-permeable membrane. These units are not restricted in any way, but can take up any configuration with respect to one another. In Fig. 4*b* is shown a similar collection of units which are now arranged in a definite sequence or pattern. If we now consider the configurational entropy of the polymer chains in an individual spherical unit, we see that in the case of Fig. 4*a* the entropy will be the same as that in Fig. 3*c* because the presence of the

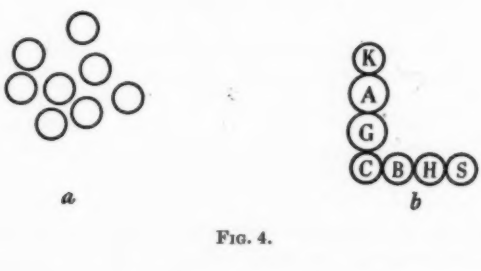

FIG. 4.

other units does not restrict the configurations in any way. But in the arrangement shown in Fig. 4*b* the restriction in the configurations of the spheres, produced by arranging them in a definite pattern, will also restrict the number of configurations of the polymer chains inside the spheres.

Quantitatively, the actual energy difference between the two types of structures will depend upon the scale upon which the arrangements are made. Any arrangements which are made on a molecular scale will require a larger supply of energy *per unit mass* than those which are effected on a larger scale.

From the considerations given above, we see that any structure involving the building up of monomer units such as amino acids into successively *higher* levels of organised structure will lead to a corresponding *decrease* in the configurational entropy of the polymer material.

III. *Real Biological Systems.*

Apart from the special case considered by Tyler (1939), we have, in the previous section, been considering hypothetical systems to which the laws of thermodynamics can be applied. These systems are all reversible systems. In a reversible change the process can be reversed by an infinitesimal change in the balance of forces operating upon the system. The system is therefore, at all stages, essentially in equilibrium.

Chemical reactions in biological systems take place at a finite rate so that reversible conditions do not exist. The building up of biological structures takes place by a complicated process of cell division in which metabolite molecules are constantly being absorbed from the surroundings, while energy sources are broken down to carbon dioxide and water and subsequently discarded ; i.e., we are dealing with an open system in which there is a constant flow of material. We cannot apply ordinary thermodynamics to open systems, although von Bertalanffy (1947) has developed a mathematical procedure which he considers to be applicable. We shall not however make use of such techniques in the present paper.

Let us now re-examine the structures shown in Fig. 4 and let us suppose for a moment that they represent living cells containing protein, nucleic acid, polysaccharide and lipid molecules. In Fig 4*a* they are considered to be unicellular organisms which do not interact in any way ; in Fig. 4*b* they are considered to represent the organised cellular structure of a differentiating organism.

Consider any of these cellular structures at a given instant in time. Now we may be able to produce a series of reversible processes whereby we could bring metabolite molecules in dilute solution first within the semi-permeable membrane of the cell by a process of isothermal compression, then into an orientated configuration by a series of compressions and extensions in the solid state. Because no process can be more efficient than a reversible process the energy changes involved would be the least that could possibly occur in the formation of these structures. In the actual biological process of synthesis, where events take place at a finite rate, the energy requirements for synthesis must be greater than they would be for a reversible process. In our hypothetical models, we have also neglected changes of internal energy in the system. The formation of polymers involves the synthesis of chemical bonds between the monomer units. The synthesis of the peptide bond—CO—NH—from the free amino acids

$$\begin{matrix} & & \mathrm{H} & & \\ \mathrm{H_2N} & - & \mathrm{C} & - & \mathrm{COOH} \\ & & \mathrm{R} & & \end{matrix}$$

is an endothermic reaction which requires a supply of energy, and the same is true for other biological polymers. The two factors of irreversibility and internal energy changes will require that all growth processes will involve a continuous supply of energy in excess of that which would be required for the entropy component in our hypothetical models. But these factors do not prevent us from calculating the entropy term in a real biological system. Fowler and Guggenheim (1939) point out that material which is not in an equilibirum state still has a definite entropy. It has been pointed out by Patat (1953) that the laws of thermodynamics cannot be applied to isolated and improbable events such as occur in biological systems. But in the particular problem with which we are concerned in the present paper, the configurational entropy of the polymer structures within

the cell, the number of units of which they are composed is very large and although the number of configurations is restricted it is still very large.

In the succeeding sections in the present paper, we shall attempt to make some purely qualitative observations on what we might expect to be the differences in energy requirements for the growth of normal cells and tumour cells, in so far as the entropy component is concerned. We cannot exclude the possibility that any difference we observe may be compensated by a difference in internal energy changes or departure from reversibility in the two cases. We may say at least, that the chemical processes in tumour cells are not likely to be more efficient than those of normal cells, if the tumour cells are produced by the action of some destructive agent.

IV. *The Growth of Tumours.*

In Fig. 5*a* we have a diagrammatic representation of the entropy changes involved in the growth of the cell. M represents the metabolite molecules in the medium surrounding the cell. These molecules are in a high entropy (low order state). A represents the corresponding condition for these materials after their

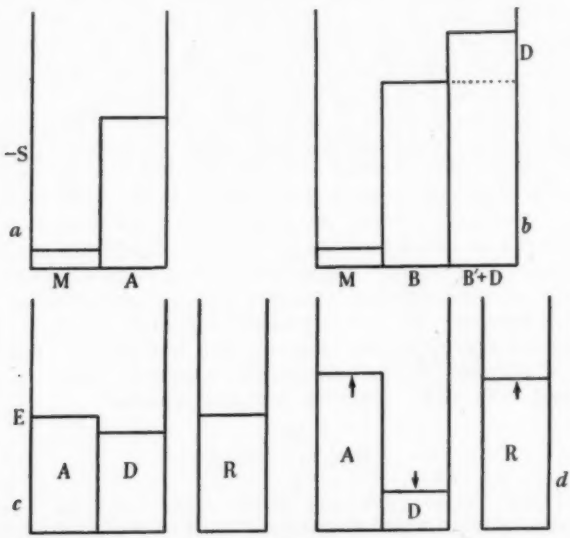

FIG. 5.

incorporation in an undifferentiated cell. In Fig. 5*b* is shown the corresponding figure for the differentiating type of cell. M corresponds, as before, to metabolite molecules. B corresponds to the entropy state of the materials incorporated within the embryonic cell. This is represented as a lower entropy than the cell A, because the cell contains within itself a molecular structure which is capable of interacting with other cells. B′ + D represents the cell when differentiated and fulfilling its proper function. D represents the further reduction

in the entropy of the whole system when the cell is called upon to carry out this function. Let us consider the possible effect of a carcinogenic agent upon the systems shown in Fig. 5. It was pointed out in Section I that the feature which might be common to the various types of carcinogen could be the ability to produce a disorganisation of the cells or tissues involved. An attack upon the cell which leads to a more random condition of its parts can lead to an increase of entropy. We might expect to find therefore that such an attack would lead to a reversal of the sequence shown in Fig. 5*a* and *b*, i.e., we should pass successively from B′ + D to B, from B to A and finally to M. The first manifestation of such a change would perhaps appear while the cell was still carrying out its function as a differentiated cell. There might be an increased entropy of the extra cellular material deposited by the cell.

An alternative way of viewing the process is given in Fig. 5*c* and 5*d*. The cell is represented as a reservoir which contains a certain concentration of material associated with its autosynthetic system, A. The work with radio isotopes suggests that active synthesis is taking place in tissues (e.g., Schoenheimer, 1949) and this applies also to highly differentiated cells. A cell will remain in an equilibrium state, or will grow, depending upon whether the rate of synthesis is equal to or greater than the rate of degradation of the structural components. Even the most highly differentiated cells can grow; the nerve cells of the body can divide at an early stage of life, but they continue to grow as differentiated cells for many years. In Fig. 5*c* the cell is considered to be in an equilibrium state, i.e., in the condition of dynamic equilibrium in a polyphase system suggested by Gowland Hopkins (quoted by Baldwin, 1947). The region D in Fig. 5*c* represents the energy requirement for the carrying out of the proper function of the cell in its differentiated form. As for the autosynthetic system, the synthesis of extra-cellular material is also associated with a condition of dynamic equilibrium. Many of the extra-cellular proteins, for example, represent unstable systems. Insulin exhibts a spontaneous denaturation in aqueous solution which appears to be due to an auto-catalytic mechanism (Waugh, 1946 ; Ambrose and Elliott, 1951). In order that a steady concentration of materials can be present in the organism, continual synthesis must occur to make up for the loss. The region D of Fig. 5*c* represents the energy requirement to maintain the steady state. The region R represents the reservoir of energy producing material in the cell, together with its associated system of enzymes.

In the normal differentiated cell all the levels of A, D and R are steady. We note that the present simple theory accounts for the existence of the steady state in differentiated organisms. In any system in which a large number of chemical reactions occur in a stepwise sequence, the products of one reaction being utilized by the next, as occurs in a series of enzymatic reactions in a unicellular organism, the growth curve should obey an exponential law (Hinshelwood, 1952), i.e., the rate of growth is proportioned to the number of cells present.

The growth of a differentiating organism does not obey this law. Various possible explanations for this difference have been suggested as for example by Rashevsky (1945) who proposes that each cell produces some substance which inhibits the growth of other cells. The entropy concept described in this paper indicates that such special mechanisms are unnecessary and that the rate of growth will decrease in relation to the rate at which the differentiation process occurs. Suppose that a carcinogenic agent acts upon the cell so that the synthesis of less

ordered material takes place. For the synthesis of a given mass of material less energy will be required if the material is in a higher entropy state. The level of energy required by the system D in Fig. 5*c* will therefore fall. This will mean that the remainder of the cell will no longer be in a steady state and the concentrations of R and of A will increase. The cell will grow. It may divide, or it may produce a giant cell. The rate of growth will be related to the extent to which the entropy of the extra cellular elements synthesized by the cell is increased. It was suggested by Haddow (1947) that tumour growth might be due to the overcoming of an energy barrier between a high energy (normal growth) and a low energy (tumour growth) state. According to the picture given above, this difference in energy arises from a change of entropy in the tissues rather than from a change of internal energy.

The conclusions of a tentative nature which may therefore be drawn as a result of applying the analysis of Section II to the problem of tumour growth are as follows :

(i) Carcinogenic agents may be those which can lead to a more random condition of the parts of the cell and tissues, corresponding to higher entropy states.

(ii) The effect of the agent will manifest itself as disorganised growth.

(iii) The process of tumour formation may be an accumulative one, possibly involving a series of step-like mutations associated with increasing degrees of disorder.

(iv) These successive stages of disorder may be associated with increasing rates of growth, owing to the increased entropy and corresponding decrease in energy requirements for synthesis of the materials produced.

V. *Experimental Evidence.*

Let us now consider the conclusions given above (Section IV), in terms of the experimental evidence.

In so far as the properties of the carcinogenic compounds themselves are concerned (Conclusion 1), it may be of interest to mention chromosome breakage. Most of the agents described in Section I can produce chromosome breakage (Darlington and Koller, 1947). This is a depolymerization process, although it is taking place on a scale much greater than those which are normally considered in polymer chemistry. In these instances at least, the carcinogens are causing an increase

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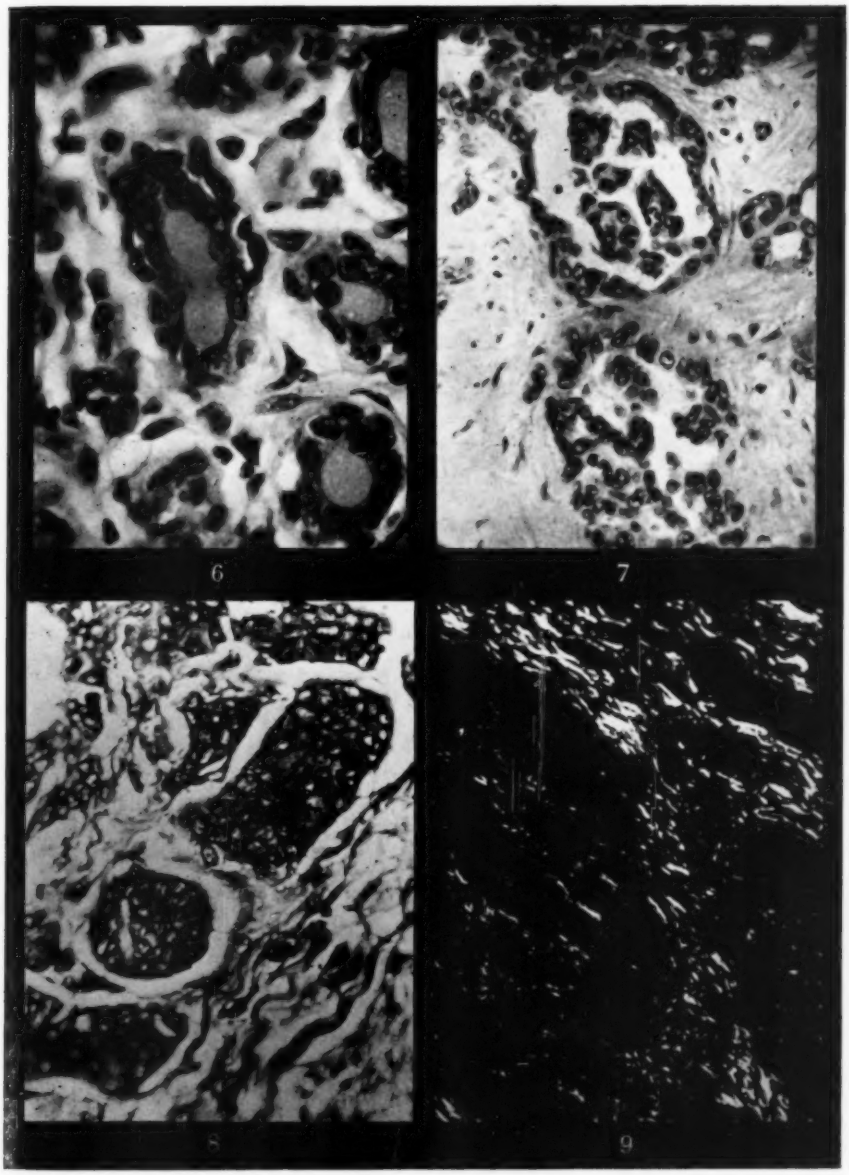
EXPLANATION OF PLATES.

FIG. 6.—Normal human breast tissue.
FIG. 7.—Human breast tissues. Early stages in loss of differentiation of duct cells.
FIG. 8.—Human breast tissue. Malignant condition.
FIG. 9.—Normal breast tissue (*polarized* light).
FIG. 10.—Wall of normal uterus.
FIG. 11.—As above in polarized light.
FIG. 12.—Myofibroma of uterus.
FIG. 13.—As above in polarized light.
FIG. 14.—Myosarcoma of uterus.
FIG. 15.—As above in polarized light.
FIG. 16.—Striated muscle fibres.
FIG. 17.—Experimental rhabdomyosarcoma.
FIG. 18.—Normal connective tissue in polarized light.
FIG. 19.—Sarcoma in polarized light. (Sarcoma region to right of field.)
FIG. 20.—Sarcoma region in unpolarized light. (High magnification.)
FIG. 21.—Breast sarcoma.



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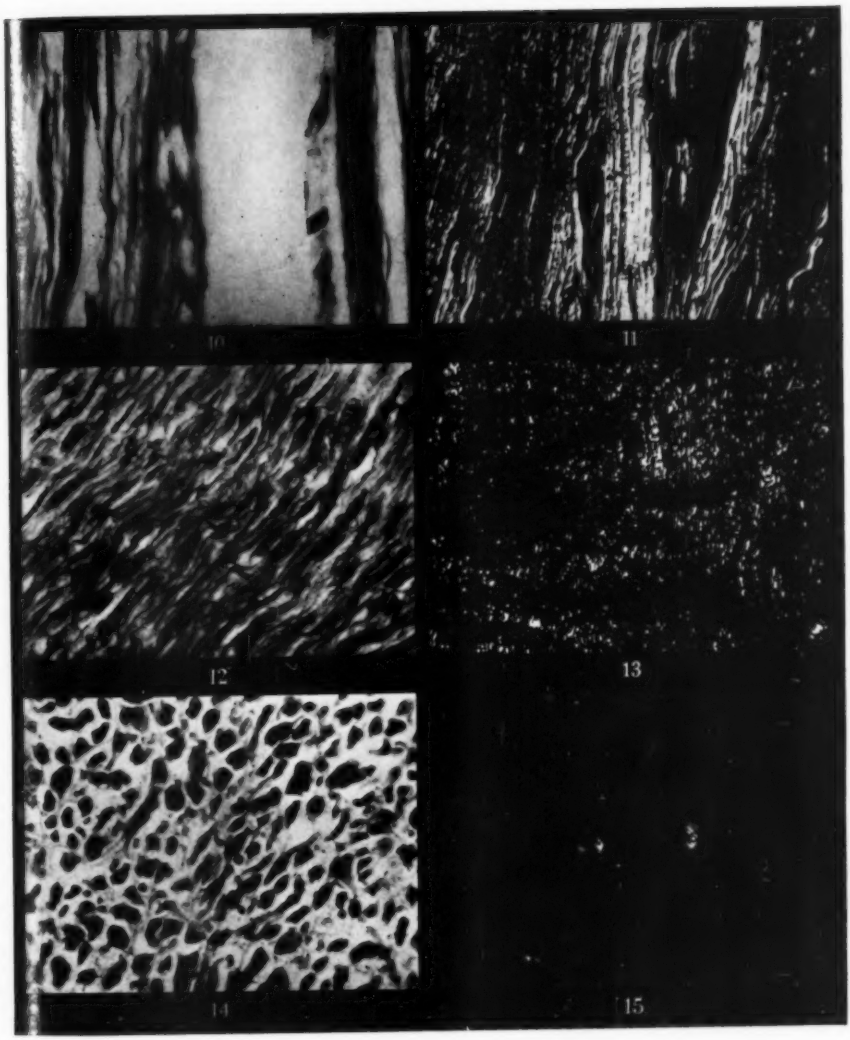
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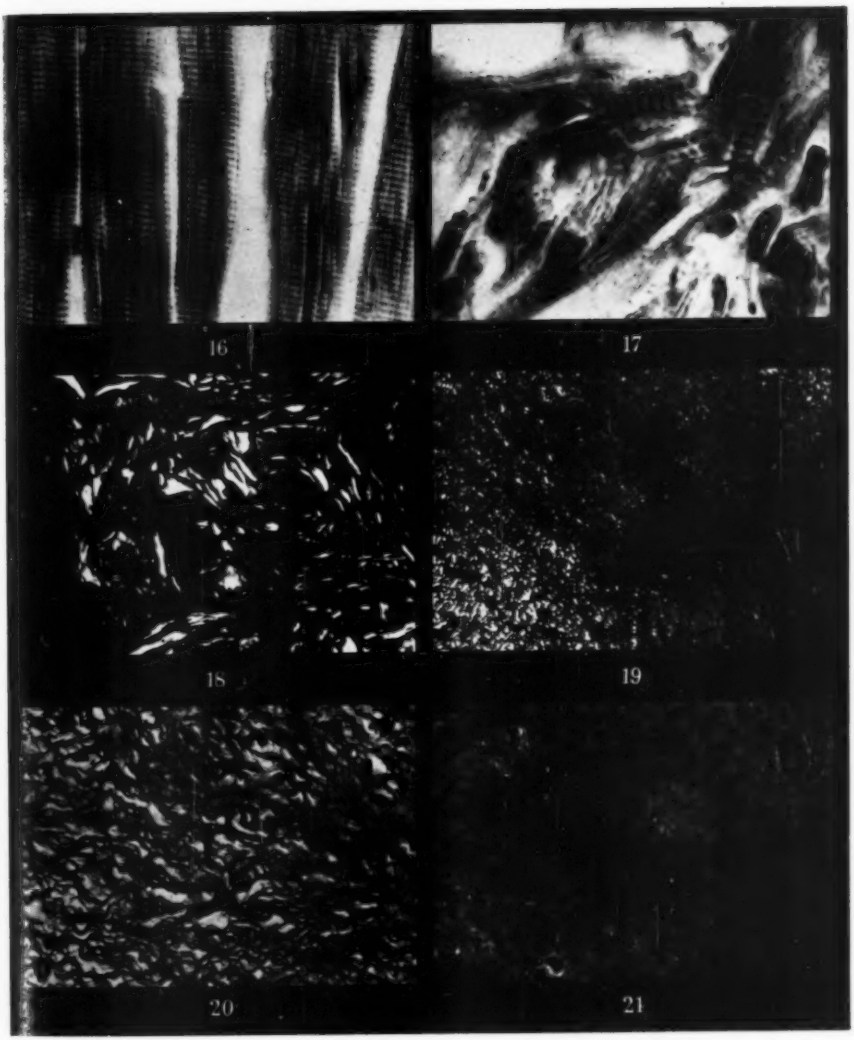

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18 19

20 21

in the entropy of the intracellular material, according to the analysis given in Section IIc. Chromosome breakage may therefore be a gross manifestation of the kind of change in the synthetic mechanism which can produce cancer cells.

Gopal-Ayengar (1953) has made an extensive study of the chromosome abnormalities which are found in normal cells and in tumour cells, particularly ascites tumour cells. The one characteristic which has been observed only in tumour cells, is the presence of supernumerary chromosomes. These are very small chromosomes, which are present in addition to the normal complement and which contain the necessary structure to enable them to be passed on during cell division. While these supernumerary chromosomes should not be looked upon as necessarily the cause of malignancy, they do perhaps provide yet another instance of a more disorganised condition of hereditary material associated with unrestricted growth. In so far as the synthetic mechanisms within the cell are concerned it has been shown by Roberts and Tishkoff (1949) that in a carcinoma of mouse epidermis, the free amino acid content is lower than that of normal and hyperplastic epidermis. Yet there is a continuous increase in the mass of protein material in the tumour suggesting, as Roberts and Tishkoff (1949) point out, " that the mechanisms for protein synthesis are much more efficient and can operate at a greatly accelerated rate even in the presence of smaller concentrations of amino acids." This condition can be understood if the entropy term is favourable for the tumour cells.

Rondoni (1939, 1942) has concluded, as a result of extensive studies of the pathology of tumours that disordered states are characteristic of these tissues.

If an increase in the configurational entropy of the synthetic mechanism in the cell has taken place, we should expect that the products of the synthetic mechanisms would show an increase of entropy. We will now examine some forms of growth which are observed in the case of tumour tissue and of the normal tissue from which they are derived. It is advisable to examine the tumours at an early stage of growth before the forms have been disturbed by secondary effects, such as necrosis, formation of fluid cavities, lack of blood supply, etc. We will first make use of the analysis as given by Tyler (1939) (Section II$a^{(2)}$).

In Fig. 6 is shown a section of human breast tissue. The ducts can be seen in cross section ; it will be seen that they are arranged in the form of hollow cylinders with the cells forming a single layer round the wall. In a case of carcinoma it is the duct cells which become malignant. An increasing amount of energy per unit mass would be required for synthesis in this case, as one passed from the solid cylinders of Fig. 8 to the longer irregular cylinders of Fig. 7 and finally to the normal ducts of Fig. 6.

As in the analysis given in Section II*b*, it would be possible to calculate the energy differences for the three forms of growth, if a reversible process for changing from one form to the other could be visualized. The actual biological processes which have taken place will not affect the result, because no process can be more efficient than a reversible process. Any energy difference which is calculated will be a minimum energy difference. We will first consider the case where the material has the least possible structure, i.e., the liquid state. The problem is analogous to the formation of a soap bubble from a solid drop of soap solution. The increase in surface area associated with the formation of the new surface requires that work must be done to provide the surface energy. In addition, a positive pressure must be applied within the bubble. The value of the pressure is proportional to the curvature of the liquid surface ($P = 2T/r$, where T is the surface tension and

r is the radius). This means that the formation of the large irregular bubble of a given wall thickness requires less energy per unit mass than does the small bubble. The formation of Fig.7 will therefore require more energy than Fig. 8, and the formation of Fig. 6 will require still more. Tissue material is not liquid, and if it were an elastic solid much more work would have to be done to form the hollow cylinder. It would be similar to blowing up a rubber tube. The work done could be calculated from the elastic constants. The deformation process is actually plastic, as was mentioned in Section II*b*. Plastic flow cannot be treated thermodynamically because it is not a reversible process, but the energy requirements in this case will evidently be intermediate between the values for the liquid and the elastic case. The successive stages in the loss of differentiation, in this case of duct carcinoma, do therefore appear to represent states of increasing entropy, at least in so far as the observed changes of form are concerned.

Phenomena of this kind are again clearly illustrated in the very complete account of the genetic origin and morphology of the melanomata by Dawson (1925). It is generally agreed that the naevi are derived from cells which would, in normal tissue, be engaged in building up a layer or sheet type of tissue. The morphology of benign melanomata is well illustrated in Plate II of Dawson. The nodular and warty forms are characteristic. In the malignant condition (Dawson, Plate B, 24–35) almost solid aggregates of tumour cells appear. We can treat the problem in a manner similar to that of the ducts of the breast tissue. The transition from a layer structure to a warty form and finally to the smooth malignant tumour represent successive stages in the reduction of surface area/unit mass for the liquid case. As in the previous example, the elastic case would require successively increasing amounts of energy for the formation of the structure corresponding to a departure from the uniformly spherical form.

We will now examine some changes associated with tumour growth, which are taking place on a molecular scale, and can be considered in terms of the analysis in Section II*c*. In Fig. 10 is shown a section of the normal tissue of the uterine wall. It consists of a highly uniform arrangement of parallel fibres. Fig. 11 shows a photograph of the fibres taken in the polarizing microscope between crossed polaroids. In this case the field is dark but the doubly refracting fibres are brightly illuminated. This is because the refractive index of the fibres measured parallel to their length is different from the refractive index measured perpendicular to their length ; the brightness of the fibres, for a given thickness, is in these pictures a measure of the degree of molecular orientation which has been achieved during the synthesis, e.g., the fibres of Fig. 11 will consist of long chain molecules arranged in parallel formation.

In Fig. 12 is shown a section of a myo-fibroma, a tumour which is usually benign. The tumour cells have been derived from the cells of Fig. 10. It will be seen that the cells are still depositing fibrous material, but it shows a distinctly wavy and irregular appearance. In Fig. 13 is shown the corresponding picture taken in the polarizing microscope. It will be seen that instead of continuous regions of birefringence, only small patches are present. It was shown, by rotating the specimen, that these are indeed patches and not due to effects produced by the wavy form of the fibres. In this case we must conclude that the fibrous material has been synthesized with a molecular structure in which only a proportion of the chain molecules lie in a parallel arrangement and that regions with irregularly coiled chains are also present. In Fig. 14 is shown an example of a

myosarcoma, a malignant growth. There is now scarcely any fibrous structure being deposited and no birefringence is to be seen (Fig. 15). The material is no longer of a fibrous and orientated character.

These results certainly suggest that the types of growth associated with successive stages of malignancy correspond to increasing disorder on a molecular scale.

In Fig. 16 are shown at high magnification normal striated muscle fibres. They are arranged in a highly regular form with the characteristic cross-banding. In Fig. 17 is shown the characteristic appearance of the material which is deposited in an experimental rhabdomyosarcoma (Haddow, Horning and Timmis, 1953). It will be seen that the muscle fibrils are deposited in an irregular manner, and actual slipping of the bands occurs in some instances. Here again the form of growth suggests that increasing randomness on a molecular scale is associated with the tumour form.

Our final example is given in Fig. 18–20. In this case we have a tumour derived from the cells which produce connective tissue. In the normal tissue the well-formed collagen fibres, which are strongly birefringent, can be clearly seen in polarized light (Fig. 18). In Fig. 19 is shown the edge of a sarcoma region in which the cells are derived from the fibroblast cells of Fig. 18. The fibroblast cells are still laying down fibrous material, but it is much more wavy in appearance, as was the case for the tumour derived from the uterine wall. The tissue was stained for collagen, using the technique of van Gieson.

The tumour showed a considerable reduction in the amount of collagen present as compared with the normal tissue. But a region shown in Fig. 20 still gives a considerable colour due to collagen. It will be seen that there is practically no detectable birefringence in the tumour region which is on the right of the field. This result suggests that there is a relative decrease in the amount of orientated fibrous material deposited in the tumour region.

In Fig 9 is shown a section of normal breast tissue, photographed under the polarizing microscope. The well-formed collagen fibrils are clearly seen surrounding each duct and filling the surrounding spaces. In Fig. 21 is shown a corresponding picture for a case where the fibroblast cells have become malignant. A fibrous structure can be seen but there is a complete absence of any birefringence.

The experimental evidence given above therefore appears to be in agreement with the general conclusion of Section IV, that tumour growth corresponds to disordered states of high entropy and that in so far as the forms of growth and molecular orientation of fibrous protein are concerned the successive stages of malignancy do in fact appear to be associated with successive increases of entropy.

It may be pointed out that a number of other instances could be quoted in favour of the general picture. The principle may help a little in understanding the relationship between carcinogenesis and growth inhibition, as first described by Haddow (1947).

If the carcinogens do in fact have a disorganising effect upon the synthetic system, it is more likely that in a given instance they will disorganise the structure to such an extent that it can no longer function at all. The limited disorganisation associated with tumour formation may be a comparatively rare event.

The substance of this paper is not intended to present yet another theory of cancer. One is certainly hesitant in attempting to apply physical methods to such complex systems but it may have helped a little if it has provided a framework

within which the intimate biochemical mechanisms associated with carcinogenesis may be expected to operate.

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ON THE CARCINOGENIC ACTIVITY OF PURIFIED CHOLESTEROL.

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THE development of the search for endogenic carcinogens has been reported in earlier publications from this Institute, (see Hieger (1949) for references to previous papers). It has been shown that the cholesterol-rich fraction of tissues from human subjects who had died of cancer or of other causes, and even commercial cholesterol, is a carcinogen. The present paper deals with the evidence for the carcinogenic activity of the purified sterol itself.

It will be useful to first present in greater detail the data for the results obtained with commercial cholesterol before it has undergone any special purification, i.e., as bought. Table I and Summary to Table I show the survival rate and tumour incidence in the first series of tests.

Some of the experiments in Table I have been reported in the 1949 paper (Hieger, 1949, p. 132, section 10*d*). They are repeated here especially to show that when this particular series of test was extended (it was carried on over the period 1944–1949) to further groups of mice, and, moreover, with different samples of commercial cholesterol, the activity of the sterol remained within the limits of uncertainty characteristic of the tests with this substance, provided other conditions remain unaltered.

All " tumours " in this paper are sarcomas at the site of injection, usually of spindle cell type. Photomicrographs are shown in the plates (Fig. 1 to 6). A good proportion of these sarcomas transplanted well. The first tumour in Table I was carried through 93 generations and the first tumour in Table IV (highly purified cholesterol) has been growing vigorously in its 25th generation. The latent period is in the neighbourhood of 19 months (Table IX). The technique of assay has been to inject 0·1–0·4 c.c. of a 10 or 15 per cent solution of the sterol subcutaneously into mice as often as is required to maintain a nodule under the skin. On the average, each mouse received of the order of 3 to 5 injections during the course of the test which lasted from the time the mice were 6–10 weeks of age until their death. In the earlier tests lard, supplied by a single firm, was used as the solvent, but in 1950 it was found that a large batch of lard from the same source had different physical properties from the lard supplied until then, and it was decided to change to olive oil as solvent, although that alteration did not guarantee that there would result a greater uniformity in the composition of the solvent at future times. It would have been advantageous from some points of view to have chosen a synthetic solvent such as tricaprylin, but since a naturally occurring fat, i.e., lard, had already been in use for a considerable time the change to another naturally occurring fat, olive oil, was considered not to be as drastic

a change in technique as would changing to a synthetic solvent. The control tests on solvents are described further on in this paper.

Purification of the cholesterol was next undertaken, as a first step using the simplest technique. Table II gives the results of tests on fractions of commercial cholesterol obtained by crystallisation from acetone. Some of these results were given in a condensed form in the 1949 paper (Hieger, 1949) referred to above; they are repeated here so that the results for the different fractions can be compared.

TABLE I.—*Tests of Carcinogenic Activity of Commercial Cholesterol (Solution in Lard).*

This table is laid out in detail in order to show the expectedly discrete and spasmodic development of tumours which appear at a low incidence in a large series of animals.

Sarcoma development at site of injection is shown by a number in parenthesis indicating the latent period in months.

Each line represents the events occurring in the same cage.

Expt. No.	Strain of mouse.	Number of mice surviving. Start of expt.	6 m.	9 m.	1 yr.	15 m.	18 m.	21 m.	24 m.	27 m.	30 m.	Sarcomas.
1	C_{57} ♂	5	5	4	$^{11}1$	1	0	–	–	–	–	1
2	C_{57}	5	5	5	0	–	–	–	–	–	–	0
3	C_{57}	6	3	3	3	3	3	3	$^{24}0$	–	–	1
4	C_{57}	6	5	4	4	3	0	–	–	–	–	0
5	CBA ♀	5	4	4	3	2	0	–	–	–	–	0
6	CBA ♂	5	5	5	5	5	2	1	0	–	–	0
7	C_{57} ♀	5	5	5	5^{13}	4	4	1	0	–	–	1
8	C_{57}	6	4	0	–	–	–	–	–	–	–	0
9	C_{57}	5	5	5	0	–	–	–	–	–	–	0
10	C_{57} ♀	5	5	5	5	5	2^{20}	1^{22}	0	–	–	2
11	C_3H ♂	5	5	4	2	1	0	–	–	–	–	0
12	C_3H ♂	5	3	3	2	0	–	–	–	–	–	0
13	C_{57} ♂	5	4	4	4	4	4	0	–	–	–	0
14	Stock ♂	5	0	–	–	–	–	–	–	–	–	0
15	,, ♂	5	3	3	3	2	0	–	–	–	–	0
16	,, ♀	5	5	5	5	2	2	1	0	–	–	0
17	,, ♀	5	3	1	0	–	–	–	–	–	–	0
18	,, ♀	5	4	4	4	$^{14}2^{16}$	0	–	–	–	–	2
19	,, ♂	5	4	4	4	3^{15}	2	1	0	–	–	1
20	C_{57} ♂	5	5	5	5	5	4	4	3	0	–	0
21	C_{57} ♂	5	4	4	4	4	2^{18}	1	1	1	0	1
22	C_{57}	3	0	–	–	–	–	–	–	–	–	0
23	C_{57}	4	4	4	4	4	4	4	3	3	0	0
24	C_{57}	4	4	4	4	4	4	4	2	0	–	0
25	C_{57} ♀	4	4	4	4	3	3	2^{23}	0	–	–	1
26	C_{57}	5	3	2	2	2	1	1	0	–	–	0
27	C_{57}	5	5	5	4	3	2^{18}	0	–	–	–	1
28	C_{57}	5	5	4	4	3	1	1	0	–	–	0
29	C_{57}	5	4	3	2	0	–	–	–	–	–	0
30	C_{57} ♂	5	5	5	5	5	5	5	4	2^{19}	0	1
31	C_{57} ♂	5	4	4	4	4	4	3	0	–	–	0
32	Stock ♂	5	5	2	1^{14}	0	–	–	–	–	–	1
33	,, ♀	5	2	0	–	–	–	–	–	–	–	0
34	,, ♂	5	2	1	1	1	1	1	1^{24}	0	–	1
35	C_{57} ♀	5	4	4	4	4	4	3^{23}	2	0	–	1
36	MRC ♀	5	5	3	3	3	3	2	0	–	–	0
37	,, ♂	5	5	4	4	3	2	2^{23}	0	–	–	1
38	,, ♂	5	5	5	5	3	3	3^{23}	2	0	–	1
39	,, ♂	5	4	4	4	0	–	–	–	–	–	0

SUMMARY OF TABLE I.—*Carcinogenic Activity of Commercial Cholesterol (Solution in Lard).*

Attention is particularly drawn to columns 12 and 13. Obviously mice which die before they reach the latent period should strictly speaking not be entered as having been at risk. The figures in Column 12, therefore, give a lower apparent incidence than is in fact the true incidence.

Strain of mouse.	Number of mice surviving.									Number of sarcomas.	Incidence of sarcomas calculated per 100 effective mice at	
	Start of expt.	6 m.	9 m.	1 yr.	15 m.	18 m.	21 m.	24 m.	27 m.		Start (%).	1 yr. (%).
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)
C_{57}	108	92	83	68	61	45	27	10	6	10	9·3	14
Stock	45	28	20	18	10	5	5	3	1	5	11	28
C³H	10	8	7	4	1	0	—	—	—	0	0	—
MRC	20	19	16	16	10	8	7	2	0	2	10	12
CBA	10	9	9	8	7	2	1	0	–	0	0	—

TABLE II.

Activity of (1) fractions of commercial cholesterol obtained by crystallising from acetone, (2) impurities in the same sample of commercial cholesterol obtained in the mother liquors from the acetone crystallisation of the acetate. These impurities still contain some cholesterol.

Expt.	Material tested.	Mouse strain.	Start of expt.	Number of mice surviving. 6 m.	9 m.	1 yr.	15 m.	18 m.	21 m.	24 m.	27 m.	30 m.	Sarcomas.	Latent periods (months).
A	Commercial cholesterol (Expts. 30–35 from Table I)	C_{57}	15	13	13	13	13	13	11	6	2	0	2	23, 19
	This test was carried out during the same period as B, C, D, E below	Stock	15	9	3	2	1	1	1	1	0	—	2	14, 24
B	Least soluble fraction from acetone crystallisation of the cholesterol used for A	C_{57}	10	9	9	8	7	6	2	2	0	—	1	18
		Stock	15	6	5	5	4	4	1	0	–	—	3	14, 18, 21
C	Moderately soluble fraction from same fractionation process which gave B	C_{57}	11	4	4	4	4	4	3	3	0	—	0	—
		Stock	16	13	13	11	7	5	2	2	0	—	1	15
D	Mother liquors from B, C fractionation	C_{57}	12	10	8	7	7	5	3	3	1	0	0	—
		Stock	15	8	5	5	5	5	2	1	0	—	0	—
E	Impurities in the same cholesterol as used in A–D, removed in the mother liquors from acetone crystallisation of the acetate (Preparation by Prof. C. W. Shoppee.)	C_{57}	11	11	11	10	9	8	6	3	0	–	0	—
		Stock	11	6	6	4	4	3	1	0	–	–	0	—

EXPLANATION OF PLATES.

FIG. 1.—Sarcoma (No. 1) induced at site of injection in a C_{57} mouse, by olive oil solution of highly purified cholesterol. × 90.

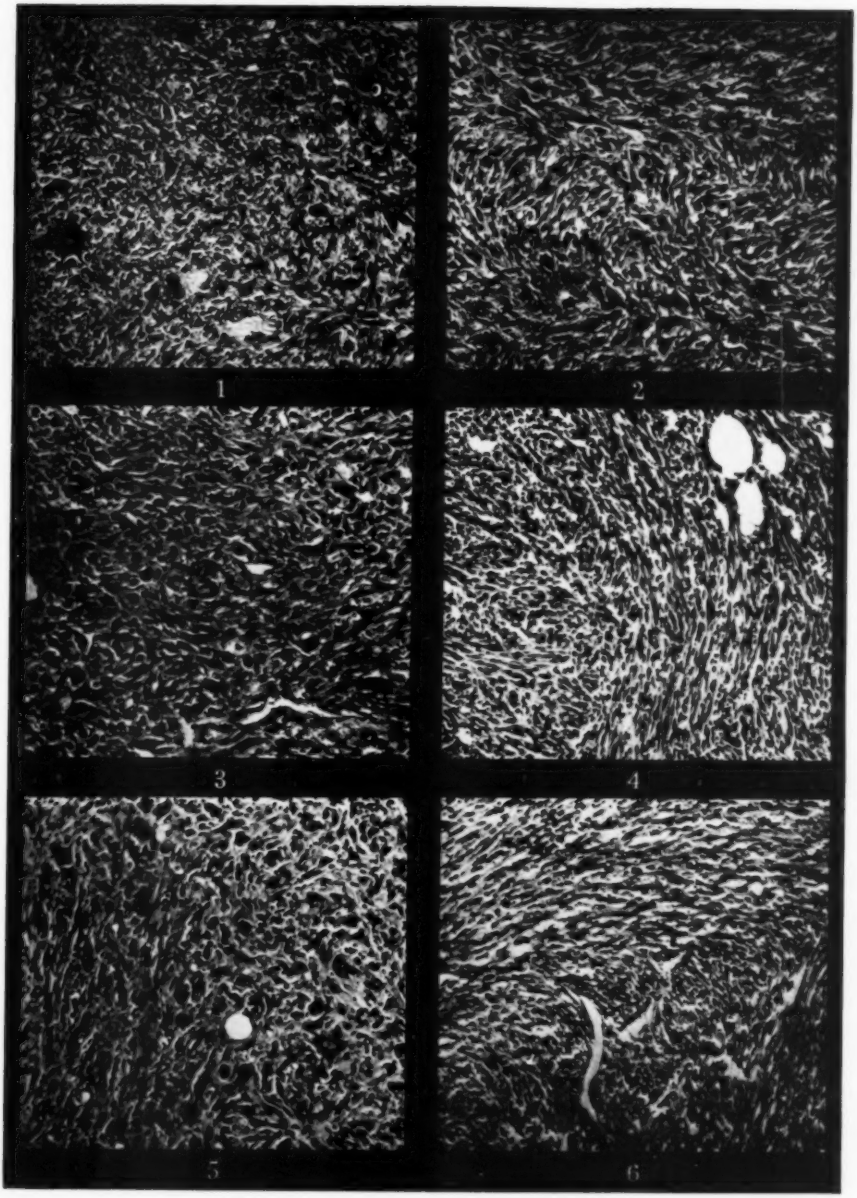
FIG. 2.—Sarcoma (No. 2) induced at site of injection in a C_{57} mouse, by olive oil solution of highly purified cholesterol. × 90.

FIG. 3.—Sarcoma (No. 3) induced at site of injection in a C_{57} mouse, by olive oil solution of highly purified cholesterol. × 90.

FIG. 4.—Sarcoma (No. 4) induced at site of injection in a C_{57} mouse, by olive oil solution of highly purified cholesterol. × 90.

FIG. 5.—Sarcoma (No. 5) induced at site of injection in a C_{57} mouse, by olive oil solution of highly purified cholesterol. × 90.

FIG. 6.—Sarcoma induced at site of injection in a C_{57} mouse by olive oil (control test). × 90.



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A sample of pure cholesterol was prepared by Professor Shoppee using the Schoenheimer technique (bromination). The results of the mouse tests are shown in Table III.

TABLE III.—*Activity of Purified Cholesterol (prepared by Prof. C. W. Shoppee).*

Strain of mouse.	Number of mice surviving. Start of expt.	6 m.	9 m.	1 yr.	15 m.	18 m.	21 m.	24 m.	27 m.	30 m.	Sarcomas.
Stock ♀	5	4	4	4	4	3	2	2	1	0	1 (27th
,, ♂	9	5	4	4	4	2	1	0	–	–	month)
C_{57} ♀	5	4	3	2	2	2	0	–	–	–	–
,, ♂	5	4	0	0	–	–	–	–	–	–	–
,, ♂	5	4	5	5	5	4	4	0	–	–	–
,, ♂	5	3	3	3	3	0	–	–	–	–	–

Cholesterol was next purified on a large scale. The method involved several stages. These were :

(1) Acetylation. This step provided several advantages, namely—
  (*a*) the OH group was protected during bromination ;
  (*b*) the acetate can be differentially eluted from Al_2O_3 by mild eluents : the unacetylated sterol when absorbed on Al_2O_3 requires more powerful eluents.
(2) Bromination, filtration and debromination.
(3) Adsorption on Al_2O_3 column and elution with benzene-pet. ether mixture.
(4) De-acetylation of the eluate by a mild saponifying agent ($KHCO_3$).
(5) Crystallisation from acetone.

The results of the mouse tests on this purified cholesterol are shown in Table IV. This experiment was carried out at our Buckinghamshire laboratories in the " Hydrocarbon-Carcinogen free laboratory "; the mice were housed in the same room as the control series (Table V) treated with the cholesterol before it had undergone purification.

Atmospheric carcinogen as a possible factor in the induction of subcutaneous sarcomas

The investigations were continued after 1949 at our country laboratories (at Pollards Wood, Buckinghamshire), which are situated 20 miles from London and could give an opportunity for carrying out this kind of work in an atmosphere probably less contaminated by carcinogens than at the Chester Beatty Institute in London where carcinogens are frequently used in different parts of the building. Experiments at Pollards Wood are conducted in rooms which have never, intentionally, contained carcinogens other than those of the sterol type. These laboratories are reached over an open court-yard, 20 yards away from other mouse rooms (referred to below as " carcinogen room "), where the mice injected with cholesterol are kept in boxes on the same racks as mice which are being treated by painting with potent hydrocarbon-type carcinogens (3 : 4-benzpyrene or dimethylbenzanthracene). By this arrangement it was possible to carry out experiments designed to test the effect of atmospheric contamination by powerful carcinogens.

There was, of course, no guarantee that contamination could have been completely avoided in the "carcinogen-free" room; for example, sawdust from a box containing benzpyrene-treated mice might accidentally be carried in to the non-carcinogen room on the shoes of anyone who had walked through the carcinogen room. However, the technicians who serviced the mice in the carcinogen room

TABLE IV.—*Activity of olive oil solution of highly purified cholesterol: Tests on* C_{57} mice.

Sarcoma development at site of injection is indicated by a number in parentheses showing the latent period in months.
Experiments at Pollard's Wood laboratory.

Start of expt.	Number of mice surviving. 6 m.	9 m.	1 yr.	15 m.	18 m.	21 m.	24 m.	27 m.	30 m.	Sarcomas.
3	3	3	3	1	1	1	1	0	–	0
3	3	3	3	3	0	–	–	–	–	0
3	3	3	3	3	3	$^{20}2$	0	–	–	1 (C_{57} ♀)
3	3	3	3	2	2	1	1	0	–	0
5	4	2	0	–	–	–	–	–	–	0
3	2	2	2	2	1	1	1	0	–	0
3	3	2	2	1	1	1	0	–	–	0
3	3	2	2	0	–	–	–	–	–	0
4	3	3	3	2	2	0	–	–	–	0
3	3	3	2	2	2	0	–	–	–	0
4	3	3	3	3	3	1^{23}	0	–	–	1 (C_{57} ♀)
4	4	2	1	1	0	–	–	–	–	0
5	5	4	4	4	4	0	–	–	–	0
5	3	2	2	2	2	1	0	–	–	0
4	4	4	4	4	4	2	1	0	–	0
4	4	4	4	4	4	3	0	–	–	0
3	2	2	2	2	2	0	–	–	–	0
4	2	2	2	2	1	0	–	–	–	0
3	3	3	3	3	3	3	0	–	–	0
3	3	3	3	2^{15}	1	1	0	–	–	1 (C_{57} ♀)
4	4	4	4	4	4	2	2	–	–	0
3	3	2	2	2	2	2	1	1	0	0
5	5	5	5	3	0	–	–	–	–	0
4	4	4	4	4	2	2	1	0	–	0
5	3	3	3	3	3	2	0	–	–	0
3	3	3	2	2	2	2	0	–	–	0
4	4	4	4	4	4	2	1	0	–	0
4	4	4	4	3	2	2	0	–	–	0
4	2	1	1	1	0	–	–	–	–	0
3	2	2	2	2	2^{18}	1	0	–	–	1 (C_{57} ♀)
4	2	2	2	2	2	2	1	0	–	0
4	4	4	3	2	0	–	–	–	–	0
4	2	2	2	2	0	–	–	–	–	0
4	4	4	4	4	1	1	1	0	–	0
5	5	5	5	5	4	4	3	2	1	0
4	4	2	2	2	2	2	0	–	–	0
4	3	3	3	3	3^{19}	1	0	–	–	1 (C_{57} ♂)
4	2	2	2	1	0	–	–	–	–	0
6	5	5	5	4	2	0	–	–	–	0
5	5	4	3	2	2	2	2	0	–	0
4	4	4	4	4	2	1	1	0	–	0
5	5	4	4	4	3	3	1	0	–	0
5	5	5	4	4	3	2	2	1	–	–
3	2	2	1	0	–	–	–	–	–	–
172	149	135	126	110	81	50	20	4	1	5

were only allowed to handle the mice in the non-carcinogen room during a fortnight (vacation) per year, when the "non-carcinogen" technician was not available.

More complete isolation of the "non-carcinogen" experiment could have been achieved by having a self-contained unit, rooms, equipment, servicing and staff completely separate from the main block of laboratories.

The results of the tests are shown in Table V.

The findings summarised in the foregoing tables suggest answers to a number of questions on the subject of this paper. For example:

Injury per se, *i.e., the pricking of the tissue on injection, as a possible factor in sarcoma production.*

The mechanical trauma caused by the needle cannot be an important factor for only 1 sarcoma appeared in over 360 mice, repeatedly injected with lard alone (control) in one series of tests and only 1 in another control series of 134 mice injected with olive oil alone. Moreover, the fatty solvent is more rapidly dispersed in the subcutaneous tissue than it is when loaded with 10–15 per cent of sterol and consequently the controls had more injections than the sterol injected mice in order to maintain a stationary nodule.

Is the carcinogenic activity of commercial cholesterol due (a) *to the sterol itself, or* (b) *to highly active impurities?*

The evidence presented in Tables IV and V provides support for (*a*), i.e., cholesterol *per se* is the true carcinogen in the commercial product.

If the incidence of sarcomas be expressed as the ratio (percentage) of tumour mice to injected mice which survived to a minimal latent period level, say 1 year, then the result can be stated thus: (*a*) Purified cholesterol gives rise to 4 per cent sarcomas in C_{57} mice, (*b*) the cholesterol before purification gave 6·4 per cent (average of 7·9 and 5·4 per cent) in stock mice; in C_{57} mice the yield was 3·6 per cent but as this figure depends upon one solitary sarcoma it would not be reliable except for the striking fact that in a number of tests C_{57} mice have repeatedly proved to be about half as sensitive to cholesterol as are stock mice (Table VI).

To assess the carcinogenic activity of a material by a number denoting the ratio (as a percentage) of tumour bearing mice to the total number of mice treated cannot be satisfactory, for obviously the mice which die before the average expected latent period cannot have had adequate exposure to the carcinogen. It would be an improvement if the assay were calculated as the percentage of tumours induced in the mice which survive to the average latent period or at least to the minimum latent period.

The data obtained from the experiments carried out with purified sterol at the Chester Beatty Institute (Table III) do not support the contention that the carcinogenesis is due to the sterol, but it should be observed that some disturbing factors appeared in this test. The mice did not survive well, and in fact the single sarcoma appeared at the 27th month in the sole survivor of the 34 mice initially injected with purified cholesterol. Twenty-seven months is near the outside limit of the range of latent periods found with the carcinogens of the cholesterol category. In an experiment with commercial cholesterol (Table II, Section A) 3 of the 4

TABLE V.—*Tumour Induction by Olive Oil Solution of Commercial Cholesterol, and of Highly Purified Cholesterol.*

Experiments at Pollards Wood laboratory.

Expt.	Mouse strain.	Location.	Number of mice at start.	Number of mice surviving. 6 m.	9 m.	1 yr.	15 m.	18 m.	21 m.	24 m.	27 m.	30 m.	33 m.	Number of sarcomas.	Sarcoma incidence calculated per 100 mice: At start (%)	At 1 yr. (%)	Latent period of sarcomas (months).
A	C_{57}	Hydrocarbon-carcinogen free laboratory	60	34	30	28	21	14	9	3	1	—	—	1	1·7	3·6	22
B	Stock	Ditto	70	45	38	38	22	18	10	2	0	—	—	3	4·3	7·9	12, 16, 24
C	,,	,,	65	63	62	57	55	41	13	11	6	0	—	3	4·6	5·4	20, 20, 29
D	,,	Carcinogen laboratory*	100	—	—	75	64	45	32	10	2	—	—	8	8	10·7	11, 12, 16, 16, 17, 20, 20, 24
E	CBA	Carcinogen laboratory*	100	—	—	71	47	45	40	29	18	5	0	2	2	2·8	19, 27
(For comparison) SUMMARY OF TABLE IV (Highly purified cholesterol).																	
F (Table IV)	C_{57}	Hydrocarbon-carcinogen free laboratory	172	149	135	126	110	81	50	20	4	0	—	5	2·9	4	15, 18, 19 20, 23

(These mice were housed in the same room and kept under the same conditions of diet, etc., as the mice in sections A, B, C above.)

* "Carcinogen laboratory." In this experiment the mice were housed in the same room and on the same racks as were mice which were being painted bi-weekly with benzpyrene or with dimethylbenzanthracene in an attempt to produce a carcinogen laden atmosphere.

TABLE VI.—*To Illustrate the Higher Response of Stock Mice, as Compared with C_{57} Mice, to the Carcinogenic Action of Cholesterol.*

The data are arranged in pairs (C_{57} : Stock — C_{57} : Stock — etc.). All the mice *within each pair* were injected with the same material, kept in the same room and fed the same diet.

Table.	Strain.	Number of mice.	Survived 1 year.	Number of sarcomas.	Sarcomas per 100 mice initial (%)	Sarcomas per 100 mice surviving 1 yr. (%)
I	C_{57}	108	68	10	9·3	14
	Stock	45	18	5	11	28
II, Section A	C_{57}	15	13	2	13	15
	Stock	15	2	2	13	100
II, Section B	C_{57}	10	8	1	10	12
	Stock	15	5	3	20	60
V, Sections A, B, C	C_{57}	60	28	1	1·7	3·6
	Stock	135	95	6	4·4	6·3

sarcomas had latent periods of 23, 24 and 29 months respectively. In the test just discussed (Table III) only 3 mice survived to 21 months, 2 to 24 months and 1 to 27 months when it developed a sarcoma. It is quite possible that the susceptible mice in this group died before the characteristic latent period had elapsed. Table IX shows the distribution of latent periods of the sarcomas discussed in this paper.

Why one mouse should develop a sarcoma while others of the same batch of similar stock or strain treated under the same conditions should not, has still to be explained. To say that the single positive mouse had the necessary degree of susceptibility does not, of course, advance the understanding of the difference. It is highly probable that multiple factors are necessary for sarcoma induction with this type of carcinogen. Seven suggest themselves, namely : susceptibility associated with strain, sex and the individual animal, environmental conditions such as atmospheric and dietetic, the " true " potency of the carcinogen and the nature of the vehicle for administration.

The effect of strain differences on susceptibility.

The summary of Table I, Table V and Table VI illustrates the effect on tumour incidence of varying the strain of mouse while keeping other factors the same. Table VI particularly shows that although tumour yield varied, the relative order of susceptibility of stock mice and of C_{57} mice remained substantially the same.

Could the injected cholesterol act as a reservoir for accumulating any circulating carcinogen, either of endogenic or of exogenic origin?

This possibility, which was put forward first by Kennaway in 1936 (unpublished) and by J. W. Orr in 1947 (unpublished), would provide an attractive theory of the mode of action of cholesterol as carcinogen. However, it would be necessary to postulate that the carcinogen is held by adsorption on the cholesterol

although still in an active stage, and that in such a condition it is not readily eluted, for clearly if it were a case of simple solubility there would be no more reason for the factor dissolving in the cholesterol than for it to be dissolved out again. Secondly injected cholesterol after a year or so in the body should be rich in absorbed carcinogen. Thirdly, if such an assumption were accepted, there would be little necessity to proceed with the question of whether pure or impure cholesterol is the true carcinogen. Fourthly, if the carcinogen which is concentrated in the cholesterol originated in the environment, it should be easy to test the hypothesis by purposely allowing the mice injected with the cholesterol to live in an atmosphere rich in carcinogen.

This test has been put into operation, and constitutes a second stage to the investigation on atmospheric carcinogens as a factor in sarcoma induction (see above).

One hundred stock mice were injected with olive oil solution of commercial cholesterol and kept in a carcinogen-rich atmosphere as described above. The control mice (135) were kept in the room free from carcinogen.

The results are shown in Table V.

Control tests on the solvents.

Lard was employed as solvent from 1936 to 1949. During this time a total of over 360 mice was tested with the solvent, lard, alone. These mice did not survive remarkably well; they gave no positive result until the series had been almost completed, when among the last few batches a single sarcoma developed. When in 1950, a change was made from lard to olive oil (hospital dispensary grade), a control series of 134 mice was set up for injection with the oil alone. A single sarcoma was found in this series. A detailed analysis of these tests is shown in Tables VII and VIII.

For the experiments described in Tables I, II, and III and Flow Sheet I lard was used as solvent. In the other experiments olive oil was used.

TABLE VII.—*Control Tests on Solvent Alone (Lard).*

One sarcoma only was obtained in these control tests on 366 mice. The "old C_{57}" mice were 12 (and upwards) months of age at the start of the experiments; their effective number should therefore be raised by a factor of the order of 2 since the initial number, 19, would represent the survivors at 12 months. The single sarcoma occurred in an old C_{57} mouse at the unexpectedly low latent period of 6 months.* However, since this mouse was 16 months of age at the start of the experiment some ageing factor for tumour induction is obviously operating. When young mice are used, the average latent period is 19 months (Table IX).

	Number of mice at start and survivors.								
	At 1st injection.	6 months.	9 months.	12 months.	15 months.	18 months.	21 months.	24 months.	27 months.
Stock . .	307	226	179	129	89	56	27	13	2
C_{57} . .	40	31	30	26	22	22	10	3	—
C_{57} (old) . .	19	13*	9	4	4	3	—	—	—

TABLE VIII.—*Tests on Activity of Solvent (Olive Oil) Alone).*
(C_{57} mice were used.)

Experiments at Pollard's Wood in hydrocarbon-carcinogen free laboratory.

Start of expt.	Number of mice surviving. 6 m.	9 m.	1 yr.	15 m.	18 m.	21 m.	24 m.	27 m.	Sarcomas.
4	4	4	4	2	1	0	–	–	–
5	5	5	4	2	2	2	0	–	–
4	4	4	4	4	2	2	1	–	–
4	4	4	3	2	2	1	0	–	–
5	4	4	4	4	0	–	–	–	–
3	2	2	2	2	1	0	–	–	–
3	3	3	3	3	2	1	0	–	–
3	2	2	2	2	2	0	–	–	–
3	3	3	3	1	1	1	–	–	–
4	4	3	3	3	0	–	–	–	–
4	3	3	3	3	3	2	0	–	–
3	1	0	–	–	–	–	–	–	–
4	2	2	2	2	2	2	0	–	–
4	3	2	2	2	2	0	–	–	–
3	3	3	3	3	0	–	–	–	–
3	2	2	2	2	2	1	0	–	–
3	2	2	2	2	2	1	0	–	–
3	3	3	3	3	3	2	0	–	–
3	3	1	0	–	–	–	–	–	–
4	4	4	4	4	3	3	0	–	–
3	3	3	3	3	2	2	2	2	–
6	2	2	2	2	2	2	2	–	–
6	6	5	3	2	2	2	0	–	1 (C_{57} ♀) at 21st mth.
5	5	4	4	4	2	0	–	–	–
6	4	4	4	4	0	–	–	–	–
6	4	4	4	3	1	0	–	–	–
6	6	5	5	3	2	0	–	–	–
6	4	4	4	4	1	1	1	–	–
6	2	1	1	1	1	0	–	–	–
6	5	4	3	3	2	0	–	–	–
6	5	3	4	2	0	–	–	–	–
134	107	95	90	77	45	25	6	2	

TABLE IX—*Latent Periods of Sarcomas Induced with Cholesterol of Different Degrees of Purity.*

Table No.	Number of sarcomas.	Latent periods (months).	Mean latent period.
I (including Section A) of Table II)	17	11, 13, 14, 14, 15, 16, 18, 18, 20, 22, 22, 23, 23, 23, 24, 24, 29	19
II (B and C)	5	14, 15, 18, 18, 21	17
III	1	27	27
IV	5	15, 18, 19, 20, 23	19
V (excluding Section F which is detailed in IV above)	17	11, 12, 12, 16, 16, 16, 17, 19, 20, 20, 20, 20, 22, 24, 24, 27, 29	19
VIII	1	21	21
	46		

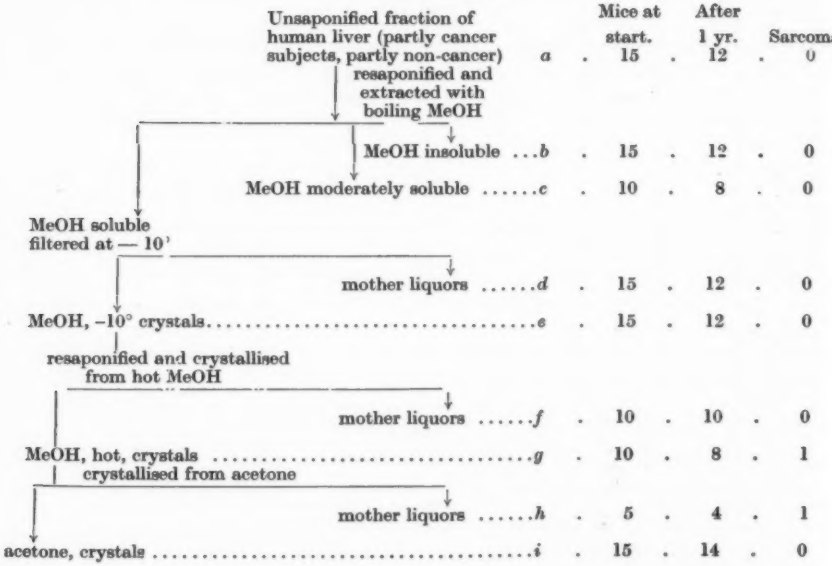
U.V. Spectroscopic Examination of Cholesterol of Various Degrees of Purity.

Key to Absorption Spectrum Curves (*Fig.* 8).

Curve 1.—*Cholesterol* purified via dibromide and recrystallised from acetic acid. (Not tested on mice).

Curve 2.—*Acetone crystallised fraction of cholesterol fraction from liver* Stage *i*, below). Liver, obtained post mortem from human subjects who had died of cancer or from some other cause was saponified and treated according to the flow sheet below.

FLOW SHEET I.


Unsaponified fraction of human liver (partly cancer subjects, partly non-cancer) a . 15 . 12 . 0
resaponified and extracted with boiling MeOH
MeOH insoluble ...b . 15 . 12 . 0
MeOH moderately soluble .....c . 10 . 8 . 0
MeOH soluble filtered at — 10°
mother liquors .....d . 15 . 12 . 0
MeOH, –10° crystals.....e . 15 . 12 . 0
resaponified and crystallised from hot MeOH
mother liquors .....f . 10 . 10 . 0
MeOH, hot, crystals .....g . 10 . 8 . 1
crystallised from acetone
mother liquors .....h . 5 . 4 . 1
acetone, crystals .....i . 15 . 14 . 0

Mice at start. | After 1 yr. | Sarcomas.

The low absorption shown by Curve 2 (low, that is, compared with the curve for the commercial product) and its inactivity, carcinogenically, suggests that treatment by crystallising from acetone removes the active fraction. However, it is difficult to explain why Fractions *a* and *e* which contained all the subsequent fractions, including *i*, did not induce tumours. When this process (crystallising from acetone) was applied on a larger scale (Table II) to commercial cholesterol it was found that the active fraction was concentrated in the crystalline product which was much more active than the mother liquors, and here the absorption curve of the acetone crystal fraction (Curve 5) showed considerably less absorption than did the cholesterol before treatment (Curve 11). Moreover, number 12 which shows a depression where Curve 5 shows a hump is the absorption curve of a carcinogenically active preparation, (2 tumours in 20 mice (initially) using cholesterol refluxed with alcoholic KOH in an experiment designed to test whether the process of saponification itself confers activity on cholesterol). Therefore, the

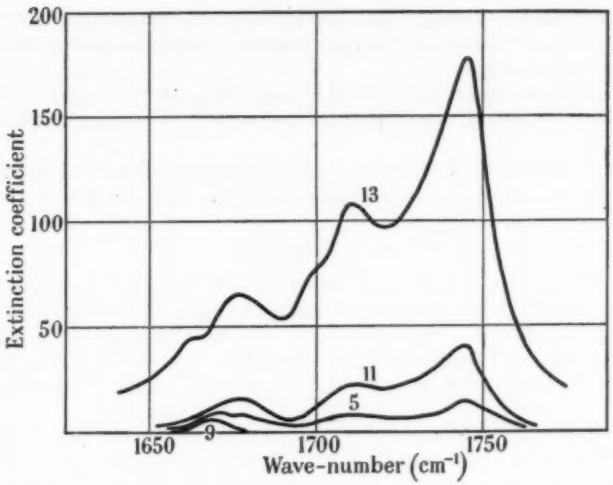


FIG. 7.—The spectra of cholesterol fractions in CS_2; all were measured in 1 mm. cell at a concentration of 25 g.p.l. Described in the Appendix.
5. Least soluble fraction from 11.
9. Pure cholesterol.
11. Commercial cholesterol: Glaxo, sample 1201.
13. Most soluble fraction from 11.

component responsible for absorption at the hump in Curve 5 (λ about 235) does not appear to be essential for carcinogenic activity. Furthermore, Curve 12 shows a hump at λ about 275–285 where Curve 5 shows a deep depression and since both preparations were active, the component responsible for this hump can hardly be essential.

Curve 3.—Cholesterol purified by bromination (Schoenheimer technique. Prof. C. W. Shoppee's preparation (see Table III.).

Curve. 4.—Unsaponifiable fraction of cream of cow's milk after crystallisation from MeOH at −10°. This preparation induced sarcomas in 2 mice from 20, initially.

Curve 5.—Least soluble fraction from acetone crystallisation of commercial cholesterol (No. BS 1201; Curve 11). (See Table II for results of mouse tests).

Curve 6.—Product from acetylation, chromatography, de-acetylation and crystallisation of commercial cholesterol. No mouse test. The experiment was designed to see how far bromination was necessary to depress the absorption of the cholesterol.

Curve 7.—Same as Curve 6 except that bromination (followed by debromination) was introduced between acetylation and chromatography. No mouse test.

Curve 8.—Commercial cholesterol as used in tests detailed in Table V.

Curve 9.—Same as Curve 7 except that different portion of the elute at the chromatographic stage was used for the subsequent stages.

Curve 10.—Large preparation of highly purified cholesterol using the combined techniques for Curves 7 and 9. See Table IV for results of animal test. The low absorption of this preparation compared with that of Curve 8 (commercial choles-

terol batch 5989) from which it was obtained and the close similarity of the carcinogenic activities of the two substances is one of the main arguments in support of the theme of this paper.

Curve 11.—Commercial cholesterol (Batch B.S. 1201) as used for some of the tests detailed in Table I (Experiments 30–35) and also for the starting out material in Table II.

Curve 12.—Commercial cholesterol after refluxing with alcoholic KOH. This preparation gave 2 sarcomas in 20 mice initially. See notes to Curve 2.

Other work on carcinogenic activity of cholesterol.

Truhaut (1947) could find no activity in his sample of purified cholesterol. Truhaut injected 20 mice, of unnamed genetic origin, with cholesterol " purifié," 20 mice with cholesterol irradiated with 10,000 r, and 20 with solvent alone, namely olive oil. A lymphosarcoma (described as " tumeur spontanée ") arose in the irradiated cholesterol group and a " huilome " in the non-irradiated cholesterol group.

One sarcoma was produced, and that in the control group of mice injected with olive oil. Truhaut's (1947) comment on this tumour is : " Il doit donc s'agir d'une coincidence."

Truhaut (1947) does not give details of the method employed purifying the cholesterol, nor of the survival rate of his experimental animals. In conclusion he states :

> " Si la susceptibilité individuelle des animaux n'est pas en cause, cela tendrait à démonter la présence, dans le cholestérol commercial, d'impuretés fortement cancérigènes qui se rapprochent vraisemblablement des substances responsables de l'activité cancérigène de la fraction des stérols précipitables par le digitonoside dans l'insaponifiable du foie de sujets cancéreux."

Steiner (1951) has also reported that no carcinogenic activity could be detected in a sample of cholesterol. He gives no details of the strain and number of mice used nor of their survival rate. He states :

> " Further studies of the carcinogenic activity in human tissue extracts have demonstrated its presence in fractions that have no ultra-violet absorption spectra characteristic of aromatic polycyclic hydrocarbons. This may indicate a new class of carcinogenic compounds.
>
> " Studies of the carcinogen potentialities of cholesterol revealed that pure cholesterol was not carcinogenic."

However, Druckrey (personal communication to the writer) has found that his sample of cholesterol, which he obtained from Windaus, did give rise to tumours on injection into mice.

DISCUSSION.

The main problem to be solved in this work is, whether the activity of the cholesterol is to be attributed to the pure sterol itself, or some companion (sterol type), or to some other carcinogen, possibly of hydrocarbon type, whether present with the cholesterol from the time of its formation or introduced later during

preparation. An examination of the absorption curves and the keys suggests that removal of the impurities responsible for absorption in U. V. does not seriously impair the carcinogenic activity. The low activity of one sample of purified cholesterol (Curve 3, Prof. Shoppee's preparation) could be explained on the poor survival rate of the mice in the test (Table III).

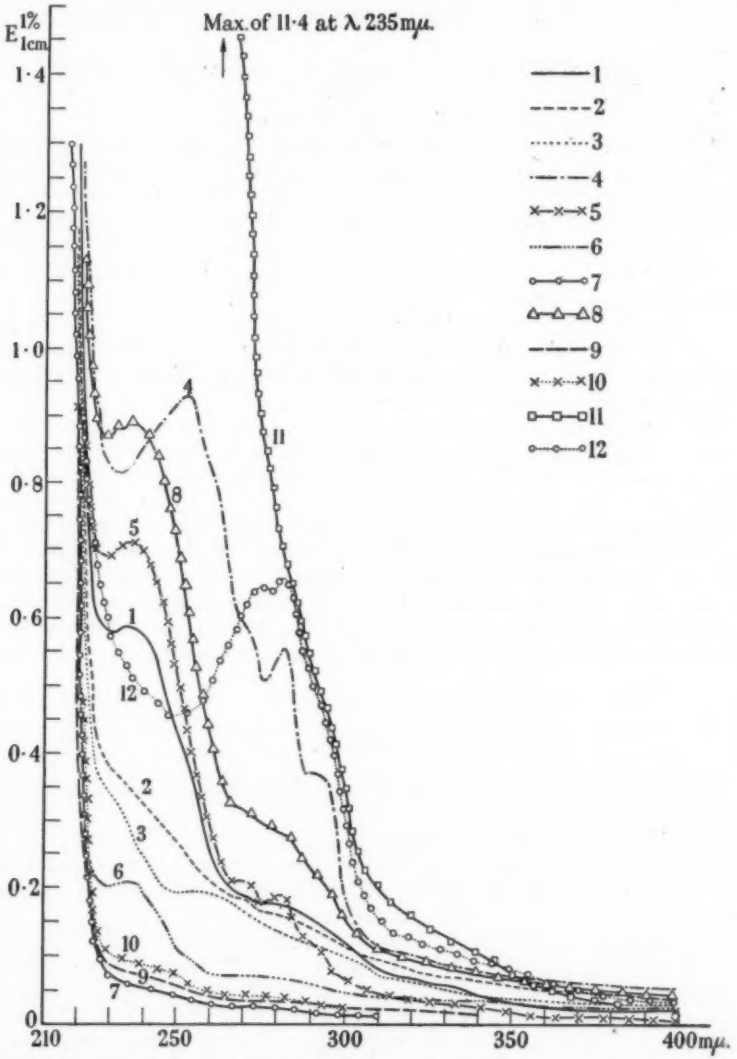


FIG. 8.—The absorption curves for the samples of cholesterol used in the tests on mice, and the effect on the absorption of different purification processes.

The tests with the commercial sample were done on stock mice and on C_{57} mice; with the pure sample they were done on C_{57} mice, on the expectation that if pure and commercial cholesterol and the control (olive oil) series were tested on the same pure line strain of mouse, more consistency would be attained. Table VI shows that stock mice are more sensitive than are C_{57} mice, hence the assayed activity of the pure cholesterol (carried out on C_{57} mice) should, theoretically, be at least as high as that of the commercial cholesterol which was carried out on stock mice.

Although the degree of activity of commercial cholesterol was much less at the Pollard's Wood laboratory than in the tests with commercial cholesterol carried out at Chester Beatty Institute (Table I) it should be noted that:

(1) These tests (Table V) were conducted in a hydrocarbon-carcinogen free room. When the test was conducted in a room where the atmosphere might be contaminated with hydrocarbon-carcinogen vapour, the incidence of tumours was about as high as the incidence found at the Chester Beatty laboratory.

(2) A first-step purification by simple acetone crystallisation of commercial cholesterol was sufficient to appreciably decrease the absorption without affecting the carcinogenic activity.

In the Chester Beatty tests (Tables I, II, III) lard was used as solvent; in the Pollard's Wood tests (Tables IV and V) olive oil was used as solvent.

(3) The part played by the solvent is not yet known. There may well be some co-carcinogenic effect between the sterol and the solvent.

(4) The responsible carcinogen may conceivably be a companion of cholesterol which has too faint an absorption (whether by reason of its low intrinsic absorption capacity or because of very low concentration) to be distinguished from cholesterol, or it may have the same spectroscopic characteristics as cholesterol. This possibility cannot be ignored but it would then require to be postulated that the companion faithfully follows the cholesterol through all the purification processes (acetylation, bromination, etc.), that it is present in the cholesterol from human tissue (cancerous human subject, as well as non-cancerous) and also in cows' milk cream and in cattle brain and spinal cord. Alternatively it would be necessary to postulate a plurality of carcinogen companions of cholesterol derived from different sources.

SUMMARY.

1. Commercial cholesterol purified by acetylation, bromination and chromatography to give a product with a very low U. V. absorption does not lose its capacity to induce sarcoma on injection, dissolved in olive oil, into C_{57} mice. Five mice developed sarcoma in a series of 172 treated mice of which 126 survived more than 1 year. In the control series injected with solvent alone, 1 sarcoma was obtained in a series of 134 C_{57} mice of which 90 survived more than 1 year.

2. The commercial cholesterol before purification induced one sarcoma in a series of 60 C_{57} mice of which 28 survived 1 year. The same cholesterol solution injected in stock mice kept under identical conditions induced 6 sarcomas in 135 mice of which 95 survived more than 1 year.

3. Data are presented showing that stock mice are of the order of twice as sensitive as C_{57} mice to carcinogenesis by commercial cholesterol.

4. The highest incidence of sarcomas obtained with commercial cholesterol

was in experiments at the Chester Beatty Institute laboratories, where sarcoma developed in 5 of 45 stock mice of which 18 survived more than 1 year.

5. The sarcomas induced by cholesterol (commercial and purified) have a long latent period. Forty-six sarcomas had an average latent period of 19 months.

6. Five hundred control mice were tested with the solvents alone (lard or olive oil). Of these 500, 249 lived more than 1 year. Two sarcomas were obtained.

The authors are deeply indebted to Dr. Joan Booth for the ultra-violet absorption spectra. Her data are an essential part of the material on which this paper is based.

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APPENDIX.

INFRA-RED SPECTRA.

The purity of the cholesterol fractions has been further followed by infra-red spectral measurements. Attention was directed to the spectra in the region characteristic of double-bond absorption, namely at 1650–1750 cm.$^{-1}$. In this region, cholesterol itself has only the one weak band at 1665 cm.$^{-1}$ due to its single ethylenic double-bond, so that the presence of carbonyl containing impurities should be easily detected due to the relatively high intensity of absorption of such groups.

From a study of the exact frequency of this band in a large number of carbonyl-containing steroids, it has been shown (Jones, Humphries and Dobriner, 1950) that a strict correlation occurs, so that from the frequency of the band, the position of the carbonyl group in the molecule can be deduced. Furthermore, it has been shown (Jones, Ramsay, Keir and Dobriner, 1952) that the intensity of absorption per carbonyl group is constant for each particular type and is independant of the rest of the molecule. From the carbonyl absorption found in cholesterol fractions, therefore, the type of impurity and its approximate amount can be deduced.

Cholesterol preparation numbers, 3, 4, 6, 7 and 9 (for key, *vide supra*) showed no appreciable carbonyl absorption (less than 0·25 per cent). Number 8 (Glaxo sample 5989) had a band at 1744 cm.$^{-1}$, assigned to a 17-ketone, equivalent to about 1 per cent of such an impurity. Number 11 (Glaxo sample 1201) had three bands at 1744, 1710 and 1674 cm^{-1}; these indicate about 9 per cent of a 17-ketone, 4 per cent of a 20-ketone (or possibly in a 6-membered ring) and 3 per cent of an $\alpha\beta$ unsaturated ketone, probably a Δ^4 : 3-ketone. Number 5, which was the least soluble fraction obtained by fractional crystallisation of this latter commer-

cial sample had the same bands but reduced in intensity to about $\frac{1}{5}$ of the original, indicating a considerable purification of the cholesterol. The most soluble fraction in this crystallisation and used for the test in Section D, Table II (number 13) contained the impurities concentrated about five-fold. The progress of this purification procedure can be followed in Fig. 7, which shows the relevant spectra.

By comparison with the findings from the ultra-violet spectra, absorption at 235 mμ appears to correspond to carbonyl absorption in the infra-red, but absorption at 251mμ, as in number 4, does not correspond to such absorption in the infra-red ; the main impurity in 4 must therefore be of non-carbonyl origin.

All infra-red spectra were measured in CS_2 solution in 1 to 3 mm. cells on a Perkin Elmer 12C spectrometer.

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THE EFFECT OF CORTISONE ON CHEMICAL CARCINOGENESIS IN THE MOUSE SKIN.

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IT is commonly believed that the genesis of tumours is closely related to the mitotic activity in the parent tissue (Willis, 1948 ; Mottram, 1944*a*, 1944*b* ; Berenblum and Shubik, 1947*a*, 1947*b*) and that agents such as croton oil which heighten mitotic activity facilitate the development of tumours. Green and Ghadially (1951), Green and Savigear (1951) and Bullough (1952) have shown that both systemic and local cortisone have a depressant effect on the mitotic activity in the the mouse epidermis. Hence it seemed worth while to try the effect of cortisone on the rate of epidermal tumour production by chemical carcinogens since a substance which diminishes mitotic activity should delay or inhibit the production of tumours.

METHODS.

Male mice, approximately 6–8 weeks old, of the WLL strain were used for all experiments. They were housed in wooden cages 12 in. × 7 in × 6 in., six to a cage, and given a diet of rat cakes, cooked flaked maize, dog-biscuits, cod-liver oil, Marmite and water, *ad libitum*. The hair in the interscapular region was removed by clipping with electric clippers.

Experiment I.

Thirty mice were prepared as described above and divided into 2 equal groups and treated as follows :

Group A (experimental : average weight at beginning of experiment 21·2 g., at end 24·3 g.) were painted twice a week with 2 drops of an 0·2 per cent solution of 9:10 dimethyl-1:2 benzanthracene (D.M.BA) in 2 per cent paraffin in acetone, and daily (except Saturdays and Sundays) with 2 drops of a cortisone suspension containing 12·5 mg./ml. over the same cutaneous area. Paintings with cortisone were commenced 7 days before treatment with carcinogen was started. Both treatments were continued until the termination of the experiment.

Group B (control : average weight at beginning of experiment 21 g., at end 26·2 g.) received exactly the same treatment as Group A except that the cortisone suspending fluid was substituted for the cortisone.

Experiment II.

Forty-five mice were divided into 3 equal groups and treated as follows :

Group A (experimental : average weight at beginning of experiment 24·8 g., at end 26·5 g.) were painted twice a week with 2 drops of a solution of DMBA (0·3

per cent) in benzene, and daily (except Saturdays and Sundays) with 2 drops of a cortisone suspension containing 12·5 mg./ml. cortisone. Cortisone treatment commenced one day before treatment with carcinogen. Both treatments were continued till the termination of the experiment.

Group B (control : average weight at beginning of experiment 21 g., at end 26·2 g.) received exactly the same treatment as Group A except that the cortisone suspending agent was used instead of cortisone.

Group C (control : average weight at beginning of experiment 21 g. at end 30·5 g.) received carcinogen paintings identical with Groups A and B, but no painting with cortisone or suspending fluid

Experiment III.

Sixty-three mice were divided into 3 groups and treated as follows :

Group A (experimental : average weight at beginning of experiment 21·25 g. at end 30 g.) were painted daily (except Saturdays and Sundays) with 2 drops of the cortisone suspension for a fortnight. At this point they received a single application of 2 drops of 1 per cent DMBA in benzene, after which no treatment was given for a fortnight. At the end of this rest period 2 drops of 5 per cent croton oil in acetone was painted in the same area twice a week for a period of 15 weeks.

Group B (control : average weight at beginning of experiment 21·5 g., at end 30 g.) received the same treatment as Group A at identical time intervals but cortisone suspending agent was used instead of cortisone.

Group C (control : average weight at beginning of experiment 21 g., at end 30·5 g.) received no treatment with either cortisone or suspending agent, the treatment otherwise being similar to that of Groups A and B.

Experiment IV.

A single application of 2 drops of 1 per cent DMBA in benzene was made to each of 43 mice. No treatment of any sort was given for the next fortnight, at the end of which period the animals were divided into 3 groups which were treated as follows :

Group A (experimental : average weight at beginning of experiment 28 g., at end 31·6 g.) were painted daily (except Saturdays and Sundays) with 2 drops of the cortisone suspension and twice a week with 2 drops of 5 per cent croton oil in acetone in the same area. Both treatments were continued till the termination of the experiment.

Group B (control : average weight at beginning of experiment 27·4 g., at end 33·1 g.) received the same treatment as Group A except that cortisone suspending agent was used instead of cortisone.

Group C (control : average weight at beginning of experiment 28 g., at end 37 g.) received no treatment with either cortisone or suspending agent but received croton oil treatment as in Groups A and B.

Tables I to IV show the rates at which tumours developed in the various groups. Tables I and II clearly show the pronounced inhibition of papilloma formation in mice receiving cortisone treatment. Thus in Experiment I no papillomata were seen compared with 15 tumours in the control group at the end of the 12th week. In Experiment II only one papilloma was observed in the cortisone-treated group while 88 and 42 tumours respectively were seen in the 2 control groups at the end of the 13th week.

RESULTS.

TABLE I.—*Showing Rate of Tumour Development in Cortisone Treated and Control Animals during Treatment with* 9:10 *Dimethyl*-1:2 *Benzanthracene* (*Experiment I*).

Time (weeks).	Number of mice surviving.		Number of mice showing tumours.		Total number of tumours.	
	Cortisone.	Suspending fluid.	Cortisone.	Suspending fluid.	Cortisone.	Suspending fluid.
5	13	13	0	0	0	0
6	13	13	0	2	0	2
7	13	13	0	5	0	6
8	13	13	0	5	0	6
9	13	13	0	3	0	4
10	13	12	0	8	0	15
11	12	7	0	7	0	8
12	12	7	0	6	0	15

TABLE II.—*Showing Rate of Tumour Development in Cortisone Treated and Control Animals during Treatment with* 9:10-*Dimethyl*-1:2 *Benzanthracene* (*Experiment II*).

Time (weeks).	Number of mice surviving.			Number of mice showing tumours.			Total number of tumours.		
	Cortisone.	Suspending fluid.	Nil.	Cortisone.	Suspending fluid.	Nil.	Cortisone.	Suspending fluid.	Nil.
5	13	14	14	0	0	0	0	0	0
6	13	14	14	0	3	2	0	3	4
7	13	14	14	0	6	6	0	8	11
8	13	14	13	0	8	6	0	15	11
9	9	14	13	0	9	7	0	26 (1)	20
10	6	14	12	1	12	8	1	44 (1)	25 (2)
11	2	11	11	1	10	8	1	52	33 (2)
12	2	11	9	1	11	8	1	80	39 (1)
13	2	11	9	1	11	8	1	88 (1)	42 (1)

Figures in brackets show number of malignant growths.

TABLE III.—*Showing Rate of Tumour Development in Cortisone Treated and Control Animals after a Single Treatment with* 9:10-*Dimethyl*-1:2 *Benzanthracene followed by Croton Oil.*

Cortisone applied during the "pre-induction" and "induction" phase but not during the "development" phase of carcinogenesis. Time calculated from commencement of croton oil treatment.

Time in weeks.	Number of mice surviving.			Number of mice showing tumours.			Total number of tumours.		
	Cortisone.	Suspending fluid.	Nil.	Cortisone.	Suspending fluid.	Nil.	Cortisone.	Suspending fluid.	Nil.
5	14	18	21	0	0	0	0	0	0
6	13	18	21	3	3	3	6	3	3
7	13	18	21	7	7	8	11	11	10
8	13	18	21	7	5	9	10	6	11
9	13	18	21	6	6	11	19	9	14
10	13	18	21	7	7	12	19	15	18
11	13	18	21	6	9	13	18	14	24
12	13	18	21	7	9	11	19	16	16
13	13	18	21	9	10	10	23	15	18
14	13	18	21	9	9	10	24	14	19
15	12	18	20	9	12	9	21	14	18
16	12	18	19	7	9	8	15	9	17

TABLE IV.—*Showing Rate of Tumour Development in Cortisone Treated and Control Animals after a Single Treatment with* 9:10-*Dimethyl*-1:2 *Benzanthracene followed by Croton Oil.*

Cortisone treatment applied during " developmental " phase of carcinogenesis. Time calculated from commencement of croton oil treatment.

Time in weeks.	Number of mice surviving.			Number of mice showing tumours.			Total number of tumours.		
	Cortisone.	Suspending fluid.	Nil.	Cortisone.	Suspending fluid	Nil.	Cortisone.	Suspending fluid.	Nil.
7	15	13	13	0	0	0	0	0	0
8	15	13	13	0	1	2	0	1	2
9	15	13	13	2	5	7	2	9	11
10	15	13	13	1	5	6	1	10	10
11	15	13	13	1	7	9	1	18	28
12	15	12	13	2	6	10	3	18	37
13	15	12	13	0	7	10	0	14	33
14	15	12	13	0	8	10	0	14	32

Experiment III was performed to test the effect of local cortisone during the period just prior to, and coincident with (i.e., the " pre-induction " and " induction " periods) a single application of carcinogen. Table III shows that under these circumstances cortisone has failed to inhibit tumour formation, a result in striking contrast with Experiments I and II.

Experiment IV was performed to test the effect of local cortisone during the " developmental " phase of carcinogenesis and the results (Table IV) clearly show that in this phase cortisone inhibits papilloma formation and the very few which formed quickly regressed.

DISCUSSION.

Results of Experiments I and II strongly suggest that cortisone applied locally suppresses papilloma formation by a powerful carcinogen. This effect is probably produced by the known epidermal mitotic depressant action of cortisone. Further, as cortisone fails to produce this effect when it is applied in the " pre-induction " and " induction " phase of carcinogenesis, no support is given to Mottram's hypothesis, that the inducing action of a carcinogen is exerted on a dividing cell. The number of cells in mitosis in the cortisone pre-treated animals during the period of " induction " would be few or none and on this hypothesis far less papillomata should have appeared whereas the numbers equalled that of the controls.

Baserga and Shubik (1954) have obtained essentially similar results. They observed that the induction of skin tumours in Swiss mice by methylcholanthrene was markedly inhibited by the administration of 0·5 mg. of cortisone daily, while Rusch (1953) found that papillomata formation by benzpyrene was much diminished by local application or feeding with cortisone. No attempt was made to determine at what stage of carcinogenesis cortisone exerts its inhibitory action, but the experiments were continued for a longer period than ours and carcinomata eventually appeared at the same time as in the controls. If confirmed, our conceptions of the proliferative " developmental " phase of carcinogenesis might have to be modified, for at present it is difficult to see how an agent can so effectively suppress papilloma production and yet have no effect on the appearance of

carcinomata. The findings of Green and Savigear (1951) may bear on this point. They showed that after 4 applications of DMB, over 14 days, the epidermal cells of the mouse ear became relatively refractory to the antimitotic action of cortisone given systematically. This was confirmed by an *in vitro* technique (Green, 1952). It might be expected therefore that chemical carcinogenesis would not be delayed or prevented by cortisone, whereas in fact the present results show that it was. A possible explanation of this apparent discrepancy is that the time of appearance of resistance to cortisone depends directly on the rate of mitosis. If this rate is strongly diminished (by cortisone) from the outset of carcinogen treatment resistance would then appear much more slowly. At the time of its appearing however the treated area of epidermis would be rich in cells in the " induction " phase of carcinogenesis since cortisone does not affect this process. Such a mass of cells, after a long exposure to the carcinogen, could be envisaged, when finally they had become completely resistant to cortisone, as emerging as a frank carcinoma. On this basis the longer the delay in starting cortisone treatment the more ineffective would its suppression of papillomata development be. Experiments to decide this point are being made.

SUMMARY.

Local treatment with cortisone, from the commencement of 9:10-dimethyl-1:2 benzanthracene painting inhibited papillomata formation in the mouse skin almost completely. Cortisone also produced this effect when allowed to act during the " developmental " phase of carcinogenesis but was quite ineffective when applied solely during the " pre-induction " and " induction " phases.

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POLYCYCLIC HYDROCARBONS IN CIGARETTE SMOKE.

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THIS paper is a full description of an investigation announced previously (Cooper and Lindsey, 1953). The recently developed methods of detection and determination of polycyclic hydrocarbons in micro-gram quantities have provided a more sensitive way of analysis than has yet been employed (Cooper, 1951, 1953; Wedgwood and Cooper, 1951, 1953). In view of the suggested connection between carcinoma of the lung and smoking (McNally, 1932; Schrek, Baker, Ballard and Dolgoff, 1950; Mills and Porter, 1950; Doll and Hill, 1950; Rigden and Kirschoff, 1952; Sadowsky, Gilliam and Cornfield, 1953; Wynder, Graham and Croninger, 1953) and the established presence of carcinogenic hydrocarbons in combustion products, it was decided to use these methods to examine cigarette smoke. The only carcinogen hitherto detected in tobacco smoke is arsenic (Gross and Nelson, 1934; Thomas and Collier, 1945; Daff and Kennaway, 1950). Recently Wynder, Graham and Croniger (1953) have induced skin carcinoma in mice by means of acetone solutions of the liquid condensed from smoke obtained by artificial smoking of cigarettes.

Preparation of Tobacco Smoke.

Although many publications have appeared upon the composition of tobacco smoke much of the work described has been on material obtained in conditions very unlike those occurring in normal human smoking. On the other hand several investigators have devised apparatus to collect smoke produced in conditions resembling closely those of normal smoking (Bradford, Harlan and Hanmer, 1936; Wenusch and Schöller, 1938; Wynder, Graham and Croninger, 1953).

The apparatus we have employed is shown in Fig. 1 and has the merit of being

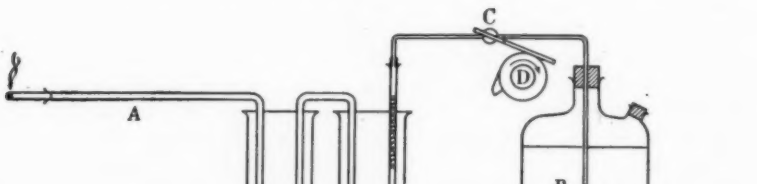

FIG. 1.—Apparatus employed.

very simple and automatic in action as well as adjustable to give variations in the conditions of smoking.

The essential parts of the apparatus are the cigarette holder and absorption tubes A made in one piece from pyrex tube of 8 mm. bore. Suction at any desired pressure was provided by the 5 l. aspirator B arranged as a Mariotte's bottle and the exit water was collected in a measuring cylinder. Thus the amount of gas drawn from the cigarette was measured. The tap C with a glass rod sealed on to it was turned on for the period necessary to simulate a " draw " made by a smoker by means of a cam attached to the periphery of a 3 inch pulley wheel, D. The glass rod was held in the groove of the pulley by means of a stretched rubber band. This wheel was driven by a gear train from a small electric motor, the speed of which was variable by adjustment of a " Variac " auto-transformer connected to the alternating current mains. Other cams could be attached to the wheel and thus the length and frequency of the " draws " were adjusted.

In the U-tubes suitable solvents were placed so that the smoke was made to bubble through them and the condensable material separated from the gas. A packing of 10 cm. of pyrex glass wool in the second U-tube served to remove the last traces of condensable matter. Both U-tubes could be surrounded by beakers full of solid carbon dioxide to assist in the condensation, but it was found that strong cooling was not always necessary to effect complete removal of the disperse phase. An examination of the habits of 150 smokers showed that, with the popular brands of cigarette sold in this country and weighing about 1·1 g. each, the average time of draw is about 2 seconds and the average time of smoking such a cigarette to a short butt (about 1·5 cm. long) is 12 minutes. These conditions are attained by our apparatus when adjusted to give a 2 second draw every 45 seconds with a negative pressure of 25 cm. water. Each cigarette used about 250 ml. air.

Smoke obtained in this way is an aerosol with a viscous liquid as the disperse phase and a gas, consisting of a mixture of unburnt air, carbon dioxide, carbon monoxide, water vapour and traces of other gases as the dispersing medium. The average amount of condensable material from 100 cigarettes weighs about 4 g. We have concerned ourselves only with the condensable disperse phase which in bulk, after the evaporation of the solvent (acetone, chloroform or cyclohexane) used to trap it, is a dark brown viscous fluid.

Preparation of Apparatus and Materials.

The solvents (cyclohexane, acetone, benzene and chloroform of reagent grade) were distilled in an all-glass apparatus, rejecting the first and last tenths. This product was then distilled through a Dufton column and collected over a range of $\frac{1}{2}$° C. leaving one-tenth as residue in the flask. The residues were not fluorescent in ultra-violet light.

All the glass apparatus employed was cleaned by immersing overnight in chromic-sulphuric acid mixture, washing and drying. A blank experiment was performed on the smoking apparatus and solvent by aspiration of 40 litres of air through the absorption tubes containing 5 ml. acetone in each U-tube. This was drained out and the absorption tube washed out with a further 5 ml. acetone. The solution was distilled to dryness in a water bath and the residue redissolved in cyclohexane. The absorption spectrum of this solution was plotted and the presence of approximately 0·02 μg. pyrene (the most easily determined hydro-

carbon in town air) established. This proportion of pyrene is negligible in comparison with the quantities found in the smoking experiments described later.

Preparation and Use of Chromatographic Columns.

The columns were prepared as described by Wedgwood and Cooper (1953) in simple glass tubes of diameter about 1 cm. with a short compressed plug of glass wool at the lower end. The alumina was poured into the tube as a thick slurry in the solvent and the tube gently tapped to ensure uniform settling.

Solutions were introduced into the column in as concentrated form as was convenient (not more than 5 ml. in volume) and the separation effected by elution with solvent. Fractions, collected by gravity, were of about 3 ml. in volume, and were examined in the S.P. 500 ultra-violet spectrophotometer. During chromatography the eluates were examined at a few definite wavelengths only and hydrocarbons were identified by specific absorption peaks and the order in which they appeared in the eluates. In quantitative work the eluates containing one compound were combined and re-examined (sometimes after additional chromatography). Fuller details of the method are given in the paper cited above.

Examination of Smoke.

It was found that the product of combustion of 50 cigarettes gave a convenient amount of material for further treatment. The dark brown solution was drained out of the apparatus and the latter washed through with more solvent into a flask. The solvent was then removed by distillation, the residue boiled twice with cyclohexane (5 to 10 ml.) and the solutions decanted off after cooling. The united cyclohexane solutions were then shaken with three quantities of 2N sulphuric acid and then with three quantities of 2N sodium hydroxide. The solution thus freed from basic and acidic substances was reduced to about 5 ml. by distillation and chromatographed on activated alumina columns. The eluates from the column were examined by the method of Wedgwood and Cooper (1953) namely by searching for absorption peaks at wavelengths known to be specific for the various polycyclic hydrocarbons. Usually the initial chromatography only revealed the hydrocarbon peaks as inflexions against considerable background absorption and the combined filtrates suspected of containing the compounds had to be passed through fresh columns in order to reveal their presence more satisfactorily. Anthracene was recognised by its peak at 376 mμ. and it was always followed closely by the appearance of a peak at 355 mμ, revealing pyrene.

The compounds were determined by making the cyclohexane solutions up to known volumes and then measuring the height of the peaks by the base line technique. For this purpose two convenient points on either side of a given peak were used to construct a base line. Calibration of the peak heights above the base line was effected by using standard curves of the authentic hydrocarbons prepared from solutions of known strength.

The background absorption interfered considerably in these experiments and various attempts were made to reduce its effect. One of the more successful methods was to evaporate off the cyclohexane and to boil the residue for an hour or so with 2N sodium hydroxide, the solution being re-extracted into cyclohexane. It is thought that much of the interference is due to esters, since some members of this class of compound have been shown to interfere in this way. It was found

that the reduction in interference was only effected when treatment with sodium hydroxide was carried out after the hydrocarbons had been separated initially by chromatography. When hydrolysis was effected on the cyclohexane extract of the whole smoke, the background absorption was even greater than before, indicating that some hydrolysis products of the smoke constituents were entering the hydrocarbon sections of the column.

The presence of pyrene and anthracene indicated in these experients was confirmed by their chromatographic behaviour relative to added methyl ethers. Relatively few compounds, absorbing light in the region studied, are associated with the polycyclic hydrocarbons on alumina columns. They are the monomethyl ethers of the hydrocarbons and certain esters. Most other organic compounds with light absorption in this part of the spectrum are much more firmly held on alumina.

Mixtures of known hydrocarbons with suitable methyl ethers in cyclohexane solution give an order of separation on the column, that serves as a standard sequence in an analysis. For example, anisole, anthracene, 1-methoxynaphthalene, pyrene, 2-methoxynapthalene and 3 : 4-benzpyrene separate in that order. The same order of elution was given with the cyclohexane solution of hydrocarbons from cigarette smoke and the hydrocarbons, anthracene and pyrene were again recognised by their characteristic peaks and determined by their peak heights. 3 : 4-Benzyprene was not detected.

Fig. 2 shows portions of the ultra-violet absorption curves of the hydrocarbons and the calibrating ethers in their chromatographic sequence. The peaks used for recognition of the various compounds are labelled with their wavelengths. It will be noted that a sequence of peaks of added calibration materials interspersed with the recognisable peaks of substances originally present, gives an infallible method of identification and determination. The results of a number of analyses utilising in all 250 cigarettes of one popular brand showed quantities of anthracene and pyrene equal to 10·2 and 9·0 μg. respectively per 100 cigarettes. Although 3 : 4-benzpyrene has not yet been detected we know that there are compounds exhibiting fluorescence in ultra-violet light present in eluates where this hydrocarbon would be expected, and we hope in due course to obtain enough material to identify them. The fact that compounds similar to benzpyrene have been found suggests that the carcinogen might also be present, possibly in quantities too small to be identified with the present scale of working, or so masked by background absorption that it has escaped detection. A considerable amount of coloured material separates on the column above anthracene and pyrene, and it is hoped that this will be resolved into its constituents in due course.

DISCUSSION.

Previous references to the presence of hydrocarbons in tobacco smoke are few. Wenusch (1937*a*, 1937*b*) and Wenusch and Schöller (1938) refer to "higher" hydrocarbons, but do not specify any definite compound. Wenusch (1934, 1935) also refers to a solid substance melting at 70–72° C. and to paraffins which he states are present in the original tobacco and are vaporised unchanged during smoking.

Acetylene has been determined in tobacco smoke by weighing it as cuprous acetylide (Fishel and Haskins, 1949). Its pyrolysis may give a ready explanation

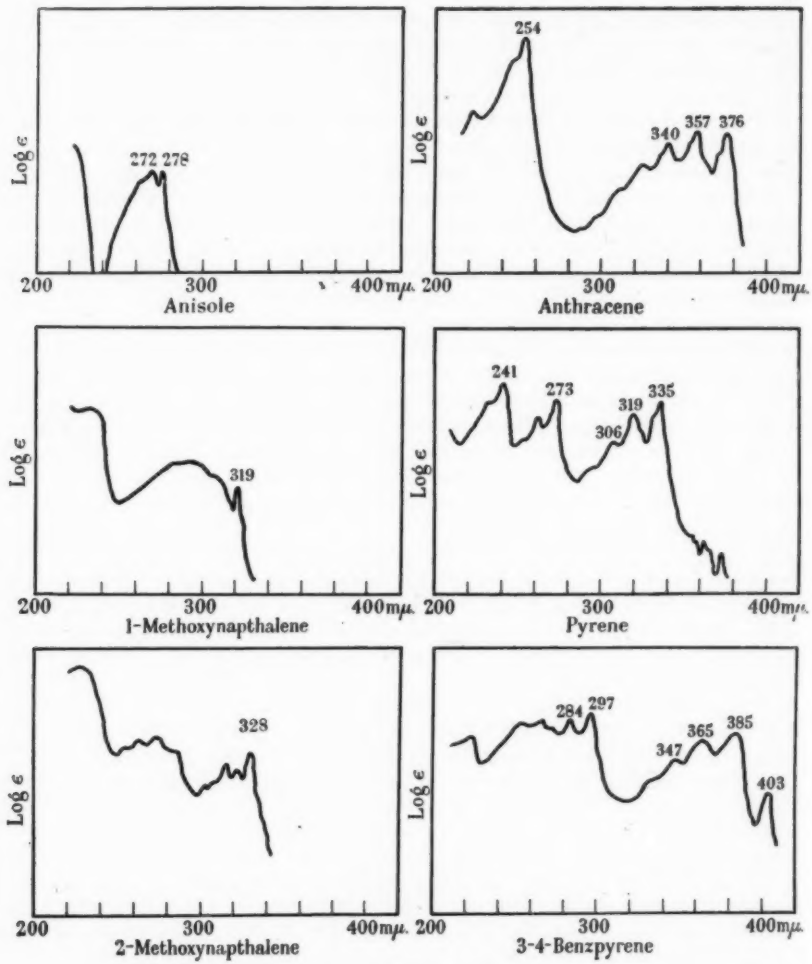

FIG. 2.—Ultra-violet absorption spectra.

of the presence of the polycyclic hydrocarbons that we have detected, and this explanation is supported by temperature measurements recorded in a later paragraph.

Azulene was obtained by Ikeda (1947) from a green oil obtained by cooling tobacco smoke, distilling the product and subjecting the distillate to chromatography.

It is of interest to consider how polycyclic hydrocarbons such as anthracene and pyrene could be formed in smoke. The presence of various unsaturated compounds has been established ; Berthelot (1886) showed that acetylene formed

tarry material upon heating, and isolated benzene and a number of polycyclic compounds from it. Kennaway (1924, 1925) has shown that strong heating of acetylene and other unsaturated materials produced carcinogenic tars at temperatures of 700° C. and above.

We have, therefore, made a study of the temperatures attained in the hot end of a burning cigarette. The use of a disappearing filament pryometer gave only approximate measure of the temperatures because the relatively small glowing end did not fill the field of the instrument. From the brightness of the glowing end during suction it was estimated that a temperature of about 900° C. was attained at the hottest part of the periphery.

More satisfactory measurements were made by using a calibrated copper-nichrome thermocouple which was threaded through the cigarette from one side to the other. After sealing the holes by means of small pieces of paper, the cigarette was smoked in the normal way and the temperature recorded from time to time. The temperature rose rapidly when the burning end advanced towards the couple and, when the glowing material was in contact with the couple, a temperature of about 650° C. was recorded. This remained almost constant while the combustion was continued round the couple and rose about 50° C. each time air was being drawn in. It thus appears that the highest temperatures during suction are confined to the surface and that the main body of the hot end is always at a temperature between 650 and 700° C. Similar temperatures were recorded when a cigarette with a thermocouple inserted was smoked in the mouth. These temperatures are sufficiently high for pyrolysis of simple compounds to polycyclic hydrocarbons. The temperatures recorded by us agree remarkably closely with those obtained independently by Wynder, Graham and Croninger (1953). Our investigations upon the composition of tobacco smoke are being continued.

The authors wish to acknowledge the helpful and kindly criticism given by Professor Sir E. L. Kennaway, F.R.S., the gift of various polycyclic hydrocarbons by Professor J. W. Cook, F.R.S., and a grant from Imperial Chemical Industries Ltd., used to purchase some of the materials and consumable apparatus used in the investigation.

SUMMARY.

1. Tobacco smoke has been obtained by smoking cigarettes in an automatic apparatus designed to simulate normal human smoking.

2. The whole of the condensable products have been extracted and the neutral extract in cyclohexane has been examined by chromatography followed by absorption spectrophotometry. The extract from 100 cigarettes showed absorption peaks characteristic of anthracene and pyrene and of heights corresponding to 10·2 and 9·0 μg. respectively.

3. The temperatures attained in the burning end of a cigarette have been measured and were found to fluctuate between 650 and 700° C. These temperatures are sufficiently high to cause pyrolysis of simpler compounds to polycyclic hydrocarbons.

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RADIOBIOLOGY OF THE C3H MOUSE MAMMARY CARCINOMA: EFFECT OF IMMUNOGENETIC FACTORS ON THE RADIOSENSITIVITY OF THE TUMOUR TREATED *IN SITU*.

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IT has been shown that the curative dose in irradiation of tumours is a relative quantity dependent upon the state of resistance of the host. Having established the median effective dose for the treatment of the C3H mammary adenocarcinoma *in situ*, it was subsequently demonstrated that when the resistance of the tumour-bearing animals was deranged by total-body irradiation, a significant decrease in the radiosensitivity of the tumour occurred (Cohen and Cohen, 1953*a*, 1953*b*). Conversely, a state of relative genetic resistance in the host can result in a marked increase in the radiosensitivity of a tumour. Oughterson, Tennant and Lawrence (1940) showed that F_2 hybrids, a cross between low-tumour and high-tumour mouse strains, bearing a mammary carcinoma originating in the high-tumour parent stock, exhibited a natural resistance evidenced by a moderate incidence of of spontaneous regressions, and were readily cured with a relatively small dose of radiation known to be almost ineffective against the same tumour in the susceptible parent strain.

In the following experiments, the curative dose for the C3H mammary carcinoma growing in reciprocal F_1 hybrids derived from high mammary tumour C3H strain and low-tumour strain mice is determined. All individuals of the F_1 generation are susceptible to transplants of the parent strain tumour and show no spontaneous regressions, yet the relative genetic incompatibility of this host tumour relationship should, if radiosensitivity is dependent on host-resistance result in a significantly lower curative dose than that required by the same tumour in the parent strain. Any similar effects attributable to the milk-factor could also be detected by comparison between the reciprocal hybrid groups.

MATERIALS AND METHODS.

Animals.

In a preliminary experiment, 80 reciprocal F_1 hybrids derived from our high mammary tumour strain C3H/Cg and a low-tumour heterogeneous albino stock, were used. Since the results of treatment in these hybrid mice were so variable as to obscure statistical comparison between the reciprocal groups, all subsequent experiments were done with reciprocal F_1 hybrids derived from the homogeneous low-tumour strain CBA/No (supplied by Dr. D. J. Nolte, Department of Zoology, University of the Witwatersrand).

For the sake of brevity, the hybrid (CBA $\times$ C3H)F_1, considered to be factor-

free, will, in this paper, be designated as FFC, and the reciprocal factor-harbouring (C3H × CBA)F_1 as FHC.

All parent strain and hybrid mice were housed in the same environment and maintained on a standard laboratory chow diet with tap water *ad libitum*. It was found expedient, in view of the temperamental breeding habits of both CBA and C3H females, to isolate routinely all pregnant mice until after the weaning of the litters. Consequently, no F_1 hybrid was ever in contact with the father, which, as has recently been shown by Peacock (1953), reduces the probability of " infection " in the FFC with milk-factor from the C3H male parent.

The incidence of spontaneous mammary tumours in old factor-harbouring (C3H × stock albino) F_1 females, which were observed for over a year and inadvertently force-bred, was at least 70 per cent. No mammary tumours were noted in the corresponding, similarly treated, factor-free females, and it was considered that the presence of the milk-factor in the C3H male parent exerted little or no influence on this group. The reciprocal hybrids of the CBA–C3H cross were observed for considerably shorter periods, during which some spontaneous tumours had already made their appearance in FHC females aged 7 to 9 months, while none had appeared in the FFC reciprocals.

Tumour.

The tumour tissue used in the following experiments consisted of established homoplasts of the C3H mammary adenocarcinoma which had not been passaged more than once or twice from spontaneous tumours arising in females of the C3H/Cg strain. In order to avoid undue variation as a result of prolonged residence in hybrid hosts, the tumour was transplanted directly from the C3H to the F_1 hybrids except, in one experiment, where a tumour growing in a hybrid was deliberately used. In each series of tumour inoculations, collateral groups of C3H mice were implanted as controls. Recipients consisted of random groups of both sexes, weighing approximately 20 to 25 g. The technique of homoiotransplantation has been described in detail in the first paper of this series (Cohen and Cohen, 1953*a*). Tumour " takes " were 100 per cent in all F_1 hybrids, with no spontaneous regressions.

Radiation procedure.

Technical factors and techniques of treatment of the tumour *in situ* used in these experiments are identical with those reported in the previous paper (Cohen and Cohen, 1953*b*). The tumour, retracted from the body onto a wax backing, is fitted into the mouth of a 2 cm. diameter open-end applicator, FSD = 25 cm., and treated at 240 kV. with no added filters, HVL = 0·34 mm. Cu., at a dose-rate of 500 r/min. in air.

Tumour size and growth rate.

The rate of growth of the implanted tumour before treatment differed sharply in the various genetic groups. Assuming, for the purpose of this comparison, an exponential increase ($V.e^{gt}$) of tumour volume (V) with time in days (t), the growth constant (g) was estimated for each mouse from measurements of tumour volume at the time of treatment, and then averaged for all animals in each genetic category. In the case of the FHC mice, the growth constants corresponded to an average

value of $g = 0{\cdot}18$ ($\pm$ 0·015), which is not significantly different from that of the C3H parent, in which $g = 0{\cdot}20$ ($\pm 0{\cdot}014$) day^{-1}. In the FFC group, however, $g = 0{\cdot}13$ ($\pm$ ·015), which differs significantly ($p = 0{\cdot}01$) from the two other groups. In the factor-free hybrid (FFC), therefore, the growth rate is significantly slower than that for the same tumour in factor-harbouring mice, whether homozygous (C3H) or hybrid (FHC). This observation corroborates Barrett and Morgan's (1949) findings that a maternal influence significantly accelerates the growth rate of the C3H tumour in F_1 hybrid mice.

The period elapsing between the appearance of a palpable "take" and the time at which the tumour was large enough for treatment by the techniques described, necessarily ranged from 10 to 60 days, and was, in general, longer in the case of FFC than among FHC mice. Further, as a result of the difference in growth rates in the two genetic subgroups, it was not always possible in this experiment to maintain a strictly constant tumour size at the time of irradiation. While the possibility that the size factor might affect the radiosensitivity cannot be entirely discounted, previous observations on the C3H host had shown that, provided the tumour was uniformly irradiated, the radiosensitivity was apparently independent of tumour size within the limits imposed by size of the treated portal.

In no treated tumour did the greatest diameter exceed 20 mm., nor the maximum thickness exceed 10 mm. Correcting for the fact that the bulk of the tumour was allowed to bulge into the open end of the applicator, it can be shown from published depth-dose tables for this quality of radiation (Braestrup, 1944) that the tumour doses range from a maximum of 111 per cent of the given air-dose at the upper pole, to an absolute minimum of 89 per cent at the base of the largest tumours treated. This difference adds little to the inherent dosimetric errors, and is insignificant in comparison to the large differences in radiosensitivity induced by the experimental variables.

All tumours were roughly spheroidal in shape, and their volumes (V) could be estimated by measurements *in situ* in the anaesthetised mice at the time of treatment, from the formula $V = \frac{\pi}{6} ab^2$, where a is the axial length and b the equatorial diameter of the spheroid. Inevitably, the average tumour-size at the time of treatment was somewhat larger in the FHC than in the FFC group, while the exceedingly skew distribution of the volumes within each group renders quantitative comparison difficult. The histograms in Fig. 1 show the reasonably symmetrical distribution in both genetic subgroups when a logarithmic transformation of the volume scale is employed. The mean-log of the volume in cubic millimeters of the FHC group is 2·12 ($\pm$ 0·08), while the mean-log-volume for the FFC group is 1·85 ($\pm$ 0·08), which is significantly smaller.

While unequivocal comparisons between the C3H and hybrid groups could be made with confidence, it was considered possible that, as a result of the variations in growth rate within the hybrid hosts, smaller tumours might be more readily cured, and the FFC group consequently biased in favour of a higher cure-rate. A more objective test of the maternal influence *per se* could, however, be obtained by eliminating from the analysis sufficient animals from the opposite extremes of the distributions, in this case the 6 largest tumours in the FHC and the 6 smallest tumours in the FFC mice (shaded areas in Fig. 1), in order to equate the geometric means of the two groups, thus permitting comparison between tumours of matched sizes.

Experimental design.

Since the object of this investigation is to determine the effect on the radiosensitivity of the parent strain tumour of genetic differences in the host, including possible maternal influences, it was necessary to estimate the magnitude and significance of differences in response of irradiated tumours in the parent strain and in the reciprocal hybrid groups respectively. Accordingly, groups of mice in the various genetic categories were treated at several dosage levels and the

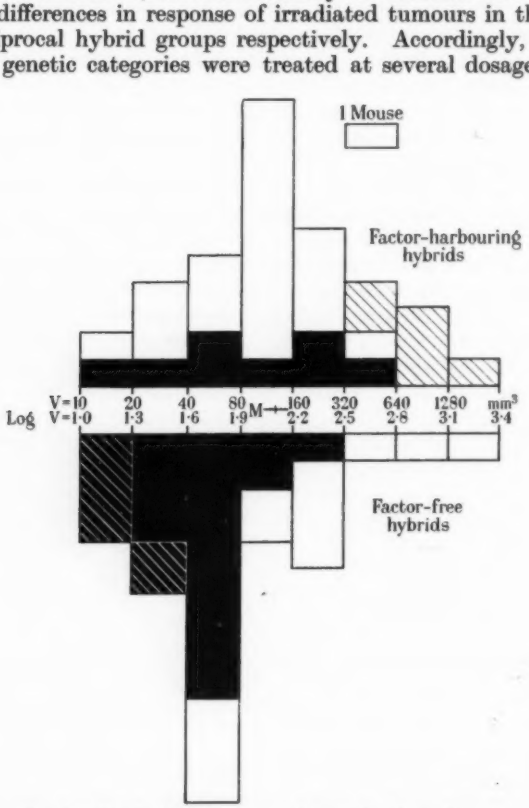

FIG. 1.—Histogram showing distribution of tumour sizes at the time of treatment in reciprocal hybrids. Black areas: Cured tumours. White areas: Non-cures. Shaded areas: Mice eliminated from statistical analysis of matched mean log-volumes. M: Common geometric mean and its standard error.

proportion of cures in each group analysed by the probit method (Fig. 2), estimating for each category, the median effective dose and its standard error, the individual variance, and the linearity or otherwise of the probit regression line.

In the preliminary experiment with the heterogeneous hybrids, the excessive individual variation and obviously non-normal distribution obscures inter-group differences and renders detailed statistical analysis unprofitable, although a rough estimate of the LD_{50} from pooled data can be obtained by inspection of Fig. 2. In the second experiment a total of 72 mice from both genetic subgroups (FHC and FFC) were irradiated at three dose levels. The proportion of cures

in each group was tabulated (Table I) and plotted on the log-probit co-ordinates of Fig. 2, together with comparative data previously determined for the parent strain. This design is analogous to a six-point assay, in which both linearity and parallelism of the regression lines can be tested. The magnitude of the parameters and the significance of the inter-group differences were computed by Finney's (1952) methods.

A third experiment was designed to determine whether the increased radiosensitivity of the tumour in F_1 hybrids was an inherent change in the tumour cells *per se*, or a reflection of host-resistance. The possibility of a change in tumour

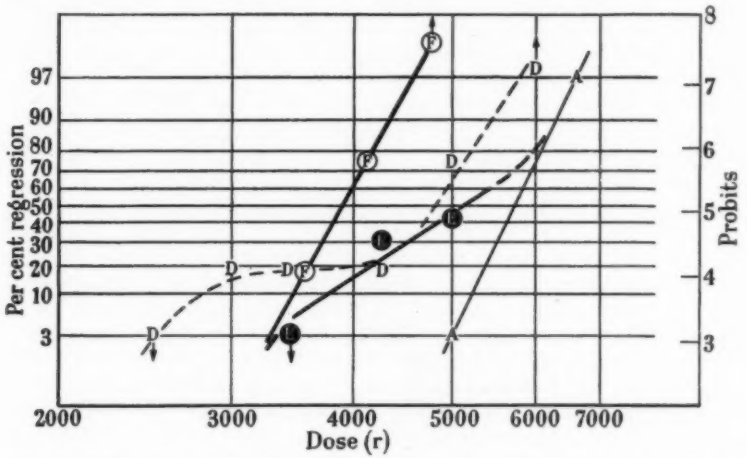

FIG. 2.—Probit diagram showing response of the C3H carcinoma growing in various situations. (A) Control series treated in homozygous C3H mice *in situ*. (D) Irradiated *in situ* in heterogeneous hybrid mice. (E) Irradiated in factor-harbouring (FHC) mice. (F) Irradiated in factor-free (FFC) mice. The increased radiosensitivity of the tumour associated with genetic diversification in the host is evident.

cells growing in a relatively alien environment was suggested by Barrett and Deringer's (1952) observation that a permanent adaptive change occurred in the C3H mammary carcinoma following one-sub-passage through susceptible F_1 hybrids. Since the object of this experiment was to demonstrate any inherent acquired radiosensitivity in the tumour, a small homoplast (first passage from a C3H mouse), growing slowly in an FFC host, which had attained a volume of only 30 mm^3. in 60 days after implantation, was excised and transplanted into mice of the three genetic categories, C3H, FHC and FFC. The donor was also re-implanted with this tumour. "Takes" were 100 per cent in all groups. When each tumour had reached a standard size, about 1 cm. in its greatest diameter, it was irradiated *in situ* with a dose of 4200 r. The re-implanted tumour in the FFC donor was also irradiated at this dose, and cured. Therefore, if the acquired radiosensitivity was a permanent adaptive change in the tumour cells capable of being carried over into the next transplant generation, it could be expected that the cure-rate of grafts originating from this tumour at the dose given would be about 75 per cent in each genetic group, or possibly even higher.

Conversely, if the resistance of the host was the deciding factor, the cure-rates in each group would be comparable with results of the preceding experiments.

RESULTS.

Preliminary experiment.

The results of treatment *in situ* at 6 different dose levels of the C3H mammary tumour growing in reciprocal F_1 hybrids derived from the C3H strain and the mixed albino stock are shown in Fig. 2, Line D. The proportion of cures with respect to dosage is based on pooled data from 80 mice, approximately equally divided between both reciprocal crosses. It is immediately apparent that the results do not follow a linear probit, thus reflecting the heterogeneity of the material. Inspection of the graph suggests that a proportion of these mice show a marked response to comparatively low doses, the median effective dose being in the region of 4500 r, which is obviously lower than that previously reported for the same tumour in the inbred parent strain. Due to the heterogeneity of the data, no evidence for the existence of a possible maternal influence on the response of the tumour could be adduced.

Second experiment.

Table I demonstrates the response to treatment *in situ* at 3 dose levels of the FHC and FFC hybrids bearing the C3H carcinoma. It will be noted that a significant proportion of cures is obtained in both reciprocal groups with relatively low dosage (4200 r) known to be completely ineffective in the C3H parent strain, the factor-free group having consistently higher cure rates at all three dose levels.

These results are plotted in Fig. 2, and the probit regression lines derived for each reciprocal group are shown. The radiosensitivity of the tumour growing in the genetically heterozygous environment of the FFC host, excluding the complicating influence of maternal factors, is shown by Line F in Fig. 2. This line is virtually parallel to that previously obtained in the parent C3H strain (Line A on the diagram), the coefficient of variation being 10 per cent. The median effective dose (LD_{50}) is found to be 3950 ($\pm$ 110*)r, compared to 5700 r previously reported in the C3H. The relative radiosensitivity of the tumour in the C3H compared to FFC mice, virtually a measure of the effect of the changed genetic milieu, can be expressed as the ratio of the two LD_{50}'s, $\frac{5700}{3950} = 1{\cdot}44$ ($\pm$ 0·05*). The magnitude of this ratio compared to its standard error shows the difference in radiosensitivity of the two groups to be highly significant ($p < 0{\cdot}0001$).

TABLE I.—*Treatment of the C3H Adenocarcinoma Growing in Reciprocal F_1 Hybrids (C3H with CBA Strains).*

Dose (r).	Factor-free hybrid (FFC). Number of mice.	Number cured.	Cures (per cent).	Factor-harbouring hybrid (FHC). Number of mice.	Number cured.	Cures (per cent).	C3H cures (per cent).
5000	8	8	100	8	3	37	8
4200	17	13	76	17	5	29	0·1
3500	11	2	18	11	0	0	0·01
Total	36	23	64	36	8	22	0·2
Total (matched volumes)	30	17	57	30	8	27	—

The corresponding data for the FHC group is shown by Line E in Fig. 2. Not only is the cure-rate, in the presence of the milk factor, considerably less than that of the factor-free mice, though still greater than that of the C3H parent, but the usual sharp response to increasing dosage has become much less sensitive, as evidenced by the considerably flatter slope of the regression line. This change is not due to fortuitous fluctuations in cure-rates, as a χ^2 test of the data reveals a highly significant departure from parallelism with the other groups. The median, effective dose for the tumour growing in FHC mice appears to be 5100 ($\pm$ 400*) r with the unusually large coefficient of variation of 25 per cent. It must be assumed, therefore, that the maternal influence is not uniform and stochastically independent of other factors. A similar result was noted in the treatment of radiation-attenuated homoplasts growing in the parent strain (Cohen and Cohen, 1954).

At the LD_{50} level, the relative radiosensitivities, evidenced by the dosage ratio between the C3H and FHC groups is 1·13 ($\pm$ ·07*), and that between the FHC and FFC groups is 1·29 ($\pm$ ·08*). The former ratio, compared with its standard error, indicates that the genetic effect *per se*, in spite of the complicating maternal influence, is probably significant ($p = 0{\cdot}05$); while the latter shows that the effect attributable to the milk factor alone within the hybrid mice is highly significant ($p < 0{\cdot}001$). The relative importance of these ratios, however, necessarily varies with the curative level tested, the maternal influence having the greater effect at doses above the median effective range.

In order to eliminate any possible bias introduced by the greater average volume of the tumours in the FHC mice compared with those of the FFC group, a small number of animals bearing tumours of extreme size were discarded in the manner previously described, so as to equate the mean log-volumes of the two groups, and the probit analyses repeated on the remaining matched tumours. This correction, however, does not alter the magnitude of the parameters or affect materially the significance of the differences between them. Further, comparison of the proportion of tumours cured within each class-interval of Fig. 1, shows the large relative preponderance of cures in the FFC group. It will also be noted (Table I) that among mice with matched mean volumes, a total of 17 out of 30 FFC's, but only 8 of 30 FHC's, were cured. This difference is significant ($p = 0{\cdot}02$).

On the other hand it is also apparent from Fig. 1 that within each genetic subgroup, the cured tumours tend to a smaller volume than the non-cures. These differences, however, are not significant, since the experiment was not designed to test the effect of tumour volume *per se* on radiosensitivity and contains insufficient data for this purpose. The question of the relative importance of genetic factors and of tumour size *per se* in determining radiosensitivity, is probably meaningless, since the smaller, more slowly-growing homoplast may itself be a manifestation of genetically-conditioned host-resistance (Eichwald, 1953). From the foregoing it may be concluded that, although the presence of the maternal factor enhances tumour growth-rates (Barrett and Morgan, 1949), and larger tumours may be less readily cured than smaller ones, nevertheless the maternal influence does affect radiosensitivity of the C3H tumour growing in reciprocal hybrids, even when the effect of gross variation in size is eliminated. Both genetic and extra-chromoso-

* Standard errors of medians and of ratios of medians.

mal factors, therefore, materially affect the response of a tumour homoplast irradiated *in situ*.

Subsequent challenge with a second inoculum in those hybrids of both reciprocal groups, previously cured of the tumour, resulted in active growth in all cases. It was also interesting to note, in the course of the experiment, that the spontaneous mammary tumours in factor-harbouring F_1 females occurred regardless of whether the animal had previously been cured or not.

Third experiment

Table II shows the results of the third experiment, in which a " radiosensitive " homoplast, growing in an FFC and later cured with 4200 r, was sub-passaged through mice of the three genetic categories and treated with the same dose. The cure-rates of these tumours in the FFC, FHC and parent C3H mice are respectively 67, 35 and 0 per cent. Testing the null-hypothesis that the radiosensitivity of the sub-passaged homoplasts is the same as in the FFC hosts (76 per cent at 4200 r, Table I), the probability of obtaining the results observed is shown in the last column of Table II. Except in the FFC hosts, this assumption is obviously invalid. Although small numbers of animals were used, the results indicate that no inherent change occurred in the tumour with respect to its radiosensitivity as a result of one passage through a relatively curable FFC host, and that the cure-rate reverted to that of the original C3H tumour in each genetic category.

TABLE II.—*Comparative Radiosensitivity in Various Hosts of the Tumour Following One Sub-passage in an FFC Hybrid.*

Recipient host.	Dose *in situ*.	Number of mice.	Number cured.	Cures (per cent).	p.*
FFC	4200	12	8	67	0·50
FHC	4200	14	5	35	$<0·01$
C3H	4200	10	0	0	$<0·001$

* p tests the hypothesis of " adaptive radiosensitisation " of the tumour (see text).

DISCUSSION.

At least four distinct biological phenomena are now known to be associated with genetic and extrachromosomal factors operating in the host-tumour relationship. The susceptibility to tumour transplantation is a simple genetic dominant, shown by the uniformly successful transfer of the inbred parent strain tumour to all F_1 hybrids. However, once such a graft is established, its rate of growth is no longer contingent on host genetics *per se*, but is dependent on the maternal extrachromosomal influence, which, when present, exerts a significantly accelerating effect. Likewise, a maternal influence, presumably the milk agent, is sufficient to induce spontaneous mammary tumours in F_1 hybrid females, the incidence approaching that in the inbred parent strain, in spite of genetic dilution. It has here been shown that the radiosensitivity of a tumour transplant, in this case the C3H mammary adencarcinoma, is dependent on both genetic and maternal (extrachromosomal) factors in the hybrid host. It appears, however, that the two factors are not necessarily independent. In the case of the factor-free F_1 hybrid, a uniform and uncomplicated response is elicited which seems to be a clear expression of immunogenetic differences between host and tumour. The

relative heterogeneity of response in the factor-harbouring F_1 is difficult to assess, but may possibly arise as a result of a variable interaction between the milk-factor and the genetically heterozygous constitution of the infant hybrid mouse.

Oughterson, Tennant and Lawrence, (1940) demonstrated the increased radiosensitivity of the parent strain tumour in F_2 hybrids where overt resistance, due to genetic segregation, was apparent. However, it has now been shown that, even in F_1 hybrids, where such gross manifestations of immunity as spontaneous regression, or failure of subsequent tumour challenges to " take " in cured animals, are absent, the principle of increased radiosensitivity in the hybrid host still holds, and may be considered as evidence of subliminal host resistance to the graft.

Barrett and Deringer (1952) and Barrett, Deringer and Hansen (1953) have described an apparently permanent adaptive change, namely an enhancement of the tumour's ability to grow in resistant backcross mice, which occurs in the C3H mammary carcinoma as a result of one transmission through susceptible F_1 hybrid hosts. Using as a source of tumour a homoplast growing in a factor-free hybrid and known to be radio-sensitive, the hypothesis that the tumour was inherently radiosensitised, as a result of this sub-passage, was tested and found to be untenable. This does not, however, exclude the possibility that prolonged serial transmission through such hosts would eventually modify the stability and radiosensitivity of the tumour; but under the conditions of this experiment, the radiocurability of these homoplasts in the three genetic categories proved to be virtually identical with the response obtained with a directly passaged C3H carcinoma. It is concluded, therefore, that the reaction of the hybrid host to the parent strain tumour is the primary determining factor. A similar phenomenon, in which host-reaction seemed to determine the radiosensitivity of the tumour, was observed when induced radiosensitivity in previously attenuated homoplasts growing in C3H mice was lost when these were transplanted to other hosts (Cohen and Cohen, 1954).

SUMMARY.

The median effective dose for the C3H adenocarcinoma growing in C3H hosts was shown to be 5700 r. The median effective dose for this tumour growing in factor-harbouring (C3H $\times$ CBA) F_1 hybrid hosts is 5100 r, and for the same tumour in the reciprocal (CBA $\times$ C3H) F_1 hybrids, presumably factor-free, is 3950 r. The differences between these groups are significant, although all three groups of mice were uniformly susceptible to the tumour and showed no overt manifestations of resistance. It appears, therefore, that the radiosensitivity of a tumour is a quantitative measure of subliminal host-resistance resulting from immunogenetic differences in the host-tumour relationship, including extra-chromosomal factors.

The mechanism involved was further elucidated when a tumour growing in a factor-free F_1 hybrid, and known to be curable with 4200 r, was sub-passaged to mice of the three genetic groups. The homoplasts responded to treatment in the same manner as the directly passaged parent strain tumour in the corresponding hosts, indicating that no inherent radiosensitisation of the tumour cells *per se* had occurred.

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RADIOBIOLOGY OF THE C3H MOUSE MAMMARY CARCINOMA: INCREASED RADIOSENSITIVITY *IN SITU* OF HOMOPLASTS ATTENUATED PRIOR TO IMPLANTATION.

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THERE are many reported examples of animals having been rendered immune or resistant, by various procedures, to subsequent inoculations of a tumour to which they are normally susceptible. The attenuation of tumour fragments by irradiation before implantation will, on occasion, produce antigenic changes in the tumour sufficient to induce immunity. Goldfeder (1942) first pointed out the quantitative relationship between the attenuation dose and the immunising property of the implant, in this case Sarcoma 180. With an adequately inbred strain like the C3H mouse, however, and using homologous mammary tumour which has not lost its genetic specificity by repeated serial transplantation, absolute resistance to further implants of the same tumour cannot be evoked.

It was previously shown that the radiosensitivity of a homologously-transmitted tumour is a function of the state of resistance of the host. When the resistance of a tumour-bearing C3H mouse is impaired by total body irradiation, the effective dose for treatment of the tumour increases; and, conversely, when some degree of resistance in a host arises from a relative genetic incompatibility of the host-tumour relationship, as in the case of hybrids bearing the parent strain tumour, the required dose is significantly decreased (Oughterson, Tennant and Lawrence, 1940). In this respect, estimation of an induced increase in radiosensitivity may be a sensitive indicator of subliminal host resistance when other criteria are lacking.

The significance of this concept was emphasised when " takes " arising from tumour fragments irradiated prior to implantation in the course of a previous experiment (Cohen and Cohen, 1953*a*) were subsequently treated and found to have a significantly increased radiosensitivity. It seemed probable that this change in radiosensitivity was a result of a minor degree of induced resistance in these animals, and a further investigation was undertaken to elucidate the respective rôles of the host and the tumour in this phenomenon.

EXPERIMENT 1.

Materials and methods.

This preliminary investigation was based on 51 mice which had been inoculated with tumour fragments, irradiated in a plastic welled slide immediately before implanatation with doses of 2000 or 2500 r, as described in our first communication (Cohen and Cohen, 1953*a*). Practically all implants attenuated at

these sublethal doses " took," after a more or less prolonged latent period averaging 30 days, and then grew progressively at the normal growth rate. When the tumours had reached the standard size of about 1 cm. in the largest diameter (overall average volume 250 $\pm$ 81 mm.^{3}), they were irradiated *in situ* with our standardised technique as previously reported in detail. All mice were individually numbered and examined at regular intervals for at least 3 months after treatment. With the doses used in this series, the tumour has, within this observation period, either disappeared completely or has resumed active growth, ulcerating the skin and killing the mouse by exsanguination or infection.

In order to detect any acquired increase in radiosensitivity, tumours arising from each attenuation-dosage group were treated at three dose levels : 3500, 4200 and 5000 r, making a total of six categories tested. Since the probability of curing an unattenuated C3H tumour in the control series with 4200 r is less than 0·1 per cent, even a few cures at this dose in an experimental series would be significant. The proportion of cures obtained at each level was tabulated and analysed by the probit method, estimating the median effective dose (LD_{50}), the slope and linearity of the regression line, and the magnitude and significance of the difference between these results and the previously established parameters for the control series.

Results.

The proportion of cures obtained in each of the six experimental categories is shown in Table I. It is fairly obvious, and readily confirmed by analysis of the data, that for any given dose *in situ* there is no significant difference between the response of tumours attenuated at 2000 r and at 2500 r. Accordingly, the pooled results from both attenuation groups, given various doses *in situ*, are shown in Table I and plotted in Fig 1 (Line G) where they may be compared with the

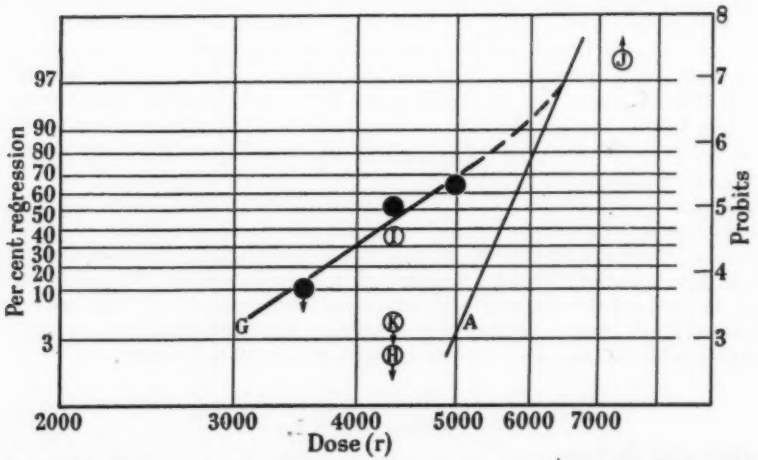

FIG. 1.—Probit diagram showing response to treatment at various doses of homoplasts of the C3H carcinoma attenuated before implantation (Line G), compared with previously established data for the unattenuated tumour (Line A). Also shown are the proportion of cures when attenuated homoplasts are transplanted to other animals (Points H, J) ; transplanted to another site in the donor mice (Point I); or recurrent in the site of surgical excision (Point K).

control series (Line A). It will be noted that the probit regression line in the attenuated series differs both in position and slope from that of the controls.

The LD_{50} for the attenuated homoplasts is found to be 4500 ($\pm$ 200*) r, compared with 5700 r previously determined in the controls, and the coefficient of variation is 26 per cent compared to 9 per cent in the controls. The *relative radiosensitivity* of attenuated tumours with reference to the controls, that is the ratio of the LD_{50}'s is $\frac{5700}{4500} = 1{\cdot}27$ ($\pm$ 0·07*). Since this ratio exceeds unity by four times its standard error, the result is highly significant ($p < 0{\cdot}0001$), indicating a definite acquired radiosensitivity of the tumour arising from an attenuated implant.

As has been previously shown, a changed radiosensitivity generally reflects an underlying change in host-resistance to the tumour. It would appear, therefore, that the attenuation procedure, while not producing any absolute immunity to further implants of the same tumour, nevertheless induces within the host, albeit the strain of origin of the tumour, a subliminal resistant state. The increased slope of the regression line compared with the control series cannot be explained by sporadic fluctuations in cure-rates, but implies a significant individual variation, presumably reflecting some irregular reaction or an inconstant degree of resistance arising in the C3H host in response to attenuated tumour fragments.

TABLE I.—*Response to Treatment* in situ *of Homoplasts (C3H Mammary Tumour) Attenuated by Irradiation Prior to Implantation.*

	Attenuation dose.					
	2000 r		2500 r			
Dose *in situ* (r).	Number of mice	Number cured.	Number of mice.	Number cured.	Total cures.	Cures (per cent).
5000	5	3	13	8	11/18	61
4200	12	7	7	3	10/19	52
3500	6	0	8	0	0/14	0

EXPERIMENT 2.

For the purpose of discerning whether the acquired radiosensitivity of attenuated homoplasts was (*a*) a persistent measurable change in the attenuated "take," (*b*) a local effect at the site of implantation or, (*c*) a manifestation of systemic resistance in the host, a second experiment was done.

Materials and methods.

Using the same mouse strain and tumours as in the first experiment, the following procedure was adopted. Tumour fragments were irradiated in the plastic slide described previously with doses of 2000 r, and then inoculated subcutaneously into the right flanks of 11 mice. Although the latent period was increased to an average of 27 days, the growth rates of the established tumours were not less than that of unirradiated implants. Each tumour was allowed to grow to a diameter of approximately 1 cm., and then excised with a sterile surgical technique and fragments

* Standard errors of median and of ratios of medians.

re-inoculated into the left flank of the tumour-bearer, and, at the same time into 2 other mice (recipients). All inoculations " took " (reverting to the normal latent interval), and there were also recurrences in 7 of the 11 surgical donor sites, giving 40 tumours in all.

Using the irradiation procedure previously described, all " donors " were treated with 4200 r *in situ*, bilaterally where necessary. Of the 22 " recipients " (2 to each donor), 11 were similarly treated *in situ* with 4200 r in order to detect persistent acquired radiosensitivity in the tumour transplants, and the remaining 11 were treated with 7500 r *in situ* to exclude the possibility of an acquired radioresistance. The former dose will result in practically no cures of unattenuated C3H mammary tumour homoplasts, so that even a few cures in the first group would be statistically significant; while with the latter dose the probability of cure exceeds 99·8 per cent, so that a few failures would indicate a significant acquired radioresistance.

Results.

At the beginning of this series of treatments, an immediate radiation mortality was noted in donors in which bilateral tumours had been treated on the same day. Since a follow-up of about 3 months after treatment is essential for evaluating cures, it was subsequently attempted to minimise the lethal effect by more careful surgical eradication of the original tumour, thus obviating the necessity for bilateral irradiation, and allowing a period of recovery of at least a week between treatments when bilateral tumours still occurred. The added disadvantage of bilateral treatments, even when the immediate mortality could be reduced, was the possibility that the increased body dose would lower the expected percentage of cures, as previously demonstrated (Cohen and Cohen, 1953*b*). The results of treatment of the attenuated homoplasts in the four experimental categories are shown in Table II, and the proportion of cures in each group plotted in Fig. 1 for comparison with the other relevant data.

TABLE II.—*Response to Treatment* in situ *of Attenuated Homoplasts after Excision and Transplantation.*

Donors:	Symbol (Fig. 1).	Dose *in situ* (r).	Number of mice.	Number cured.	Cures (per cent).	Normally expected cure rate (per cent).
Recurrent in site of excision	K	4200	7	0	0	0·1
Transplanted in opposite side	I	4200	11	4	37	
Recipients I	H	4200	11	0	0	
Recipients II	J	7500	11	11	100	99·8

In spite of the adverse effects of bilateral irradiation, it can be seen in Table II that a significant proportion of cures (37 per cent) did eventuate in the case of the *re-inoculated* tumours in the donors treated with 4200 r. From Fig. 1 it can be seen that the proportion of cures to be expected at this dosage with unattenuated homoplasts (Line A) is exceedingly small, while in animals bearing attenuated implants (Line G) it is 40 per cent, from which this result (Point I) does not differ appreciably. On the other hand, the combination of body dose and damage of the vascular stroma by surgery may be considered responsible for the sharp reduction

in cure-rate of the tumour recurrences in the excision sites (0/7 cures), even where increased radiosensitivity of the tumour on the other side existed. These findings eliminate the possibility that the increased radiosensitivity is due to a local resistance arising during the sojourn of the attenuated homoplast at the original implantation site, but indicate the existence of a generalised or systemic change.

Reference to Table II also shows that, when the attenuated "take" was transplanted to other recipients, the resultant tumours were neither more radiosensitive (0/11 cures at 4200 r), nor less radiosensitive (11/11 cures at 7500 r) than the unattenuated control homoplast in the C3H mouse (Cohen and Cohen, 1953*a*). In this connection it is interesting to note that, although the original tumour fragments attenuated with 2000 r prior to implantation exhibited the prolonged latent interval of 27 days, the latent period of the subsequent transplants of these "takes," both in the donors and in the new recipients, was the same as unattenuated tumour (7 to 15 days). These observations indicate that, in so far as its growth rate and radiosensitivity in new hosts are concerned, the previously attenuated homoplast has apparently recovered to a large extent from the initial irradiation.

DISCUSSION.

The most significant finding in these experiments is that some degree of resistance arises in a proportion of C3H mice as a result of the inoculation of a viable mammary tumour fragment, attenuated with a known sublethal dose of X-rays prior to implantation. Aside from the prolonged latent period before such an attenuated implant becomes palpable (Goldfeder, 1947), no other gross evidence of alteration as a result of such a procedure has ever been previously reported for this type of host and tumour. This subliminal resistance, or change in the host-tumour relationship, is manifested by an increase in the radiosensitivity of the attenuated "take." It is of interest to note that the response of radiation-attenuated homoplasts in the C3H mouse closely resembles, both in the magnitude of the effective dose and in the individual variance, that of unattenuated C3H tumour growing in factor-harbouring (C3H $\times$ CBA) F_1 hybrids (Cohen and Cohen, 1954). The attenuation procedure, therefore, seems to induce a degree of resistance, presumably a similar antigenic difference between host and tumour, at least as great as that engendered by the tumour growing in F_1 hybrids harbouring the milk factor.

The following facts concerning the nature of this phenomenon have been elucidated :

(1) While not excluding the possibility that the tumour cells have been inherently changed by this procedure, it is obvious that any such change is not, in itself, sufficient to affect the radiosensitivity of the transplanted attenuated "takes" in the environment of a new host. Conversely, there is no evidence of acquired radioresistance in the tumour as a result of previous irradiation, in conformity with Montgomery and Warren's findings (1953).

(2) It is unlikely that any local change in the tumour bed contributes to the changed radiosensitivity since no cures were obtained when recurrences following the local excision at the original site were treated. It can probably be assumed, too, that damage to the tumour bed by the surgical excision was sufficient to abrogate the effect of systemic resistance.

(3) Since the increased radiosensitivity persists even where the tumour has

been transplanted to another site in the original donor, it must be concluded that the effect is due to a systemic, generalised resistance mechanism.

The phenomenon of induced resistance to experimentally transmitted cancer has been extensively investigated. In the past, results in this field were often contradictory and confusing (Woglom, 1929), due to the use of heterogeneous strains of animals and gentically alien tumours. Under these circumstances, absolute immunity to tumours can be readily induced by a variety of experimental procedures. There are also reported instances of absolute immunity to a transplanted tumour being induced in the strain of origin, even where the animals are genetically homogeneous (Gross, 1943 ; Goldfeder, 1945 ; Lewis and Aptekman, 1952 ; Foley, 1952). In each of these reports, however, the tumour had been repeatedly subpassaged for many generations before its immunising ability was tested. The long transplantation history of these and other commonly used tumours, maintained solely by serial transmission, is surveyed by Dunham and Stewart (1953). It is generally realised that such procedures may permit antigenic diversification or mutation in the tumour, which, in effect, results in a relatively unstable host-tumour relationship. Consequently, many of these findings are largely invalidated in so far as their applicability to human cancer therapy is concerned.

It would appear that only when a supply of spontaneous tumours is constantly available within a given strain, and is routinely transplanted through no more than a few subpassages to experimental animals of the same strain, can the host-tumour relationship be considered adequately stabilised. Where such stringent conditions prevail, absolute immunity to a tumour cannot be evoked (Bittner, 1936 ; Barrett, 1940 ; Lewis, 1940 ; Fardon and Prince, 1953 ; Foley, 1953). A recent review by Hauschka (1952) contains an extensive analysis of the immunogenetic complexities of host-tumour relationships used in experimental cancer research.

With respect to the C3H mammary carcinoma, the authors have shown that, even where some degree of immunogenetic difference existed between the tumour and the host, as in the case of F_1 hybrids bearing the parent strain tumour, no overt-immunity could be elicited by curing the comparatively radiosensitive tumour *in situ* (Cohen and Cohen, 1954), or by previously inoculating the animals with radiation-attenuated fragments (unpublished data).

When one correlates these facts with the findings of the foregoing experiment, it becomes evident that resistance to tumour is by no means an " all-or-none " phenomenon. It seems likely that partial or subliminal states of resistance can be induced in the stable host-tumour relationship which, while not obviously detectable by conventional methods, nevertheless yield significant results in the form of a perceptible increase in radiosensivity.

In this connection, it is significant that Gorer (1947) demonstrated high titres of circulating anti-tumour iso-antibodies in mice dying of foreign strain tumour transplants, which grew progressively in spite of antigenic differences with the hosts. This form of apparently ineffective resistance may be identical with that responsible for the enhanced response to radiotherapy, from which one might postulate that iso-antibodies may be elicited by the attenuation procedure even where obvious genetic differences between host and tumour do not exist. It is suggested that an efficient method of rendering homologous tumour antigenic to its host would have a potent radiosensitizing effect.

SUMMARY.

C3H mice bearing homoplasts of the C3H mammary adeno-carcinoma, which had been attenuated prior to implantation with 2000 r and 2500 r, were treated *in situ*. The LD_{50} of these tumours was found to be 4500 ($\pm$ 200) r, which is significantly lower than that of unattenuated homoplasts (5700 r).

The increased radiosensitivity was not a local phenomenon, but persisted in the original hosts even where the established attenuated homoplast was excised and transplanted to another site. When the previously attenuated homoplasts were transplanted from the original hosts to new hosts, however, the curative dose reverted to that of unattenuated tumour.

It is considered probable that the inoculation of a viable tumour fragment, attenuated by sublethal doses of radiation prior to implantation, induces a state of subliminal resistance in the host which enhances the radiosensitivity of the resultant tumour.

Facilities for the maintenance of our experimental animals were generously provided at the South African Institute for Medical Research by Dr. J. F. Murray, to whom we are deeply indebted.

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STUDIES ON RADIOSENSITIVITY AND "IMMUNIZING" ABILITY OF MAMMARY TUMOURS OF MICE.*

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In a series of earlier investigations it was shown that two transplantable mammary adenocarcinomata, one which arose in a mouse of the C3H strain and the other in a mouse of the DBA strain, differed significantly in their biological and physiological properties, as evidenced by their rate of growth, metabolic activity, and radiosensitivity (Goldfeder, 1947; Goldfeder and Cameron, 1948; Goldfeder, 1950*a*, 1950*b*, 1951). A significant number of mice of both C3H and DBA strains, in which either previously irradiated tumour grafts failed to produce tumours or established, actively growing tumours regressed following irradiation, became resistant to viable grafts of the respective tumours (Goldfeder, 1950*a*). However, it was not known whether these two tumours had been serially transplanted in mice genetically identical with those in which the tumours originally developed, as the mice were obtained from various laboratories (Goldfeder, 1950*a*). The mice used for transplantation undoubtedly came from different sublines of these two strains. Therefore, additional experiments were carried out in an attempt to elucidate the following questions:

1. What is the underlying factor or factors responsible for the difference in the radiosensitivity of mammary tumours?

2. Is it possible to induce an immune state in homozygous mice to autogenous tumours by inoculation of these tumours attenuated by irradiation?

3. Does the regression of a well-developed transplanted tumour following irradiation *in vivo* render the animal resistant to the development of spontaneous tumours?

This study was made possible by the use of the irradiation technique (Goldfeder, 1950*a*) which facilitates the exposure of the tumour and protects the rest of the body of the animal from secondary scattered radiation, thus permitting the treated animals to live their life span. Previous work on the irradiation of tumours has been summarized by the writer (1945*a*). There is no doubt that results obtained with tumours heterogenous to the animal hosts—that is, tumours which have a different genetic constitution from that of the host, are not comparable with those obtained with tumours autogenous to the animal hosts—that is with tumours having the same genetic constitution as that of the host, or with, those in patients suffering from cancer. As will be seen, the results obtained in animal experiments, using hosts and tumours of the same genetic derivation are comparable with observations frequently noted in human cancer patients.

* A preliminary report of this investigation was presented at the Annual Meeting of the American Association for Cancer Research, April, 1952, (Goldfeder, 1952).

MATERIAL AND METHODS

C3H mice for breeding purposes were supplied by Dr. J. J. Bittner with the following information : " The mice we have sent you have been of pure lines tested to be homozygous ; they bear the milk agent and have an incidence of spontaneous tumours of over 90 per cent." Several pairs of DBA breeders were obtained from Dr. T. C. Hauschka, of the Cancer Institute in Philadelphia, who stated : " The Lankenau Hospital Research Institute originally obtained DBA mice from the Jackson B. Memorial Laboratory on July 5, 1933, and they were designated at that time as inbred strain ' D '. These mice have been inbred since : they behave immunologically like DBA/212, and have an incidence of over 85 per cent of spontaneous mammary tumours among breeding females. Mean tumour age 8 months ; range from 6–12 months ; based on a total of 62 breeding females."

Mice of both the C3H and DBA lines were bred by brother to sister matings in the writer's laboratory. Purina laboratory chow and water *ad libitum* constituted the basic diet. Mature mice also received lettuce or carrots twice weekly and sunflower seeds occasionally ; the nursing mothers received bread soaked in milk. Spontaneous tumours developed in 7–12-month-old females of both C3H and DBA strains.

A spontaneous mammary tumour of a 9-month-old C3H female mouse was excised under aseptic conditions. The edges of the tumour were cut with a sharp scalpel into pieces about 2 mm. in diameter and transplanted into 8 6-week-old mice. Palpable tumours appeared in all 8 mice within 14 to 16 days following transplantation.

Several cross-sections of the primary tumour were fixed in Zenker's fluid and stained with Harris haematoxylin and eosin. The histological appearance of the tumour was that of an adenocarcinoma Type B, according to the classification of Dunn (1945) (Fig. 1). This tumour has been carried in serial transplants in C3H mice for the past 4 years. There has been 100 per cent of " takes " and no instance of spontaneous regression. A tumour of about 5 mm. in diameter usually develops from an implant within a period of 12 to 14 days, and grows progressively.

A spontaneous mammary tumour of an 11-month-old female mouse of the DBA strain was excised aseptically. This tumour was firmer in consistency than that removed from the C3H mouse described. Fragments of about 2 mm. dissected from the edges of the tumour were implanted subcutaneously into 10 DBA mice (8 males and 2 females). Tumours of about 5 mm. in diameter appeared in all grafted mice within 16 to 18 days.

Cross-sections of the original tumour were fixed and stained by the same procedure as that used for the C3H tumour. This tumour according to Dunn (1945) represented an adenocarcinoma Type A (Fig. 2). It has been carried in serial transplants in mice of the DBA strain for more than 4 years. Its rate of growth is rather constant. Implants usually develop into tumours of measurable size within 12 to 16 days.

The spontaneous mammary tumours arising in mice of both strains (C3H and DBA) are of different types: Haemorrhagic, cystic, or solid, and varying in their degree of differentiation. Individual mammary tumours, not only in different mice of the same strain, but multiple tumours in the same mouse, may vary in their biological characteristics, as shown by Strong and Little (1920). Since the

above-mentioned properties play a rôle in the radiosensitivity of tumours, the tumours used in the present experiments are therefore specifically designated C3HB and DBAH.

The investigation on the radiosensitivity of these tumours started after the eighth serial passage of both tumours.

The procedure of irradiation of tumour grafts *in vitro* was as follows: The edges of the tumour were cut into pieces of about 2 cm. in diameter which were then divided into two parts. One part was used for implantation into control mice, while the other was exposed to X-radiation. The tumour pieces used for irradiation were spread on a No. 1 round coverslip $\frac{7}{8}$ inch in diameter, which had been previously attached by a drop of sterile water to a mica sheet and covered with a Maximow depression slide, in the concavity of which moist filter paper had been placed to maintain the water content of the tissue. The tissue was exposed at 12·5 cm. target-tissue distance from an oil-cooled pulsating potential Coolidge tube. The radiation factors were: 200 kV, 20 mA, 0·5 mm. Cu plus 1·0 mm. Al filtration; HVL of 1·1 mm. Cu. The dose rate was 602 r/min. measured in air ($\pm$ 5 per cent). The absorption of radiation by the cover glass, mica sheet and Maximow slide was about 10 per cent, which was taken into consideration in calculating the X-ray dose delivered to the tumour tissue. Immediately after irradiation the tumour fragments were implanted into about 2–3-month-old mice, most of which were litter mates. In two additional experiments the mice were injected intradermally with aliquots of irradiated tumour cells, suspended in physiological saline. This was done because, according to observations made by several investigators, the intradermal route of immunization appears to produce more effective and lasting results (Besredka, Magat, Laval and Besnard, 1936; Kolmar and Tuft, 1941). For the latter experiments the tumour fragments following irradiation were cut with sharp curved scissors until an almost homogeneous mass was obtained. A dense cell suspension was made in sterile 0·85 per cent saline solution (about 10,000 cells/c. mm.). This cell suspension was injected intracutaneously in 0·05 c.c. doses by means of a tuberculin syringe and 25 gauge needle in two or three sites of the abdominal wall of each mouse.

The latent period and the number of "takes" produced by tumour grafts irradiated *in vitro* served as criteria of the effect of a given dose of X-radiation.

The procedure of irradiation of the established tumours *in vivo* was as follows: Actively growing tumours ranging in size from 1·0 to 1·5 cm. in diameter were exposed to filtered X-radiation while the rest of the animal organism was shielded by a lead chamber (Goldfeder, 1950*a*). During the early experiments the tumours were irradiated *in vivo* under the same physical conditions as the tumour grafts *in vitro*. During the later experiments, the following physical factors were employed: The radiation was generated by a Westinghouse "Quadrocondex" Constant potential unit at 200 kV, 15 mA; the incident beam was filtered by 0·5 mm. Cu plus 1·0 mm. Al; HVL equalled 1·0 mm. Cu; FSD, 20·0 cm.; field size, 12 cm. in diameter. The dose rate measured in air by a thimble ionization chamber during the course of the investigations varied from 395 r/min. to 402 r/min. ($\pm$ 5 per cent). Special measurements were made with and without the lead shield, the ionization chamber being placed in the same position. The measurements indicated no significant scattering from the lead shield. Since the tumours were relatively much smaller than the field size they were exposed to a practically even distribution of radiation. Because of the sizes of the tumour (1·0 to 1·5 cm.

in diameter) and HVL (1·0 mm. Cu), the back-scatter amounted to 5 per cent. The X-ray doses were applied to the tumours in a single exposure. Measurements of the tumours (length, width, and thickness) with calipers were made every second day or twice weekly. Complete regression of the irradiated tumours and the survival time of the hosts 4 months thereafter without the appearance of new growth, served as criteria for evaluation of the response of the tumours to a given dose of filtered X-radiation applied *in vivo*. In addition, histological studies were made of sections taken from some primary irradiated tumours and from secondary tumours which developed within 2 to 3 weeks following irradiation of the primary tumour either in the inguinal or axillary regions or on the edges of the exposed areas.

To determine the minimum X-ray dose producing complete regression of the tumour, preliminary experiments on a small number of mice were carried out and followed by irradiation of a larger number of tumours for better statistical evaluation.

RESULTS.

A. *Observations on C3HB Transplantable Mammary Tumour.*

The results obtained on implantation of the irradiated C3HB tumour-grafts are recorded in Table I are presented graphically in Fig. 3. The first retardation of tumour growth, as indicated by a slight increase in the latent period of tumour appearance, was produced by a dose of 2000 r. The increase of the X-ray dose

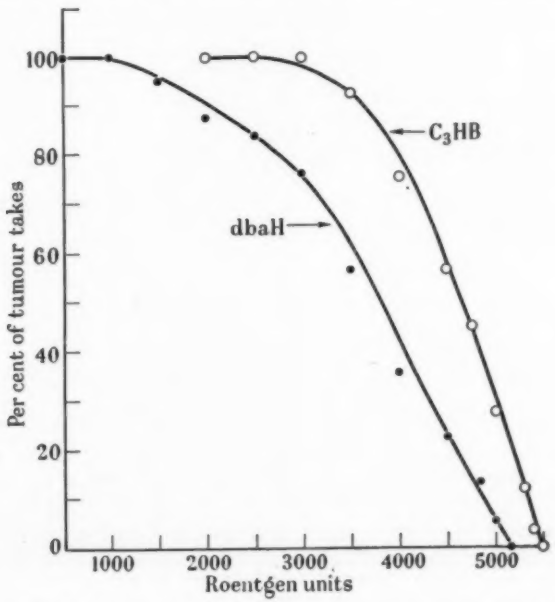


FIG. 3.—Typical curves showing the percentage of tumour " takes " (C3HB and DBAH) after various doses of radiation.

produced an increase in the latent period and a decrease in the number of " takes." A sudden drop of " takes " occurred after a dose of 4000 r. Only 4 per cent of the implants produced tumours after exposure to 5400 r. No tumours were produced by implants exposed to a dose of 5500 r.

In Experiment No. 11, an implant irradiated with 5000 r developed a tumour after a latent period of 117 days. In this, as well as in other similar instances, the question arises whether the tumour cells had been attenuated by X-radiation to such an extent that they remained dormant for this comparatively long time, or whether only a few cells had escaped injury and took, therefore, a much longer time to develop a palpable tumour. This question cannot be answered with any certainty. It is certain, however, that the tumour grew on the site of grafting and that the rate of growth and the histological pattern were similar to those of a tumour developed by a non-irradiated implant.

In no instance did a tumour, developed by an irradiated implant, regress spontaneously, contrary to observations frequently made when the tumour implant and the host are not of the same genetic origin.

Search for induced resistance

The mice in which the irradiated tumour grafts failed to produce tumours (Experiments 7-14, Table I) were reimplanted with fresh, viable tumour grafts. Due to the long latent period, the second implantation into mice of this series was performed 3 to 4 months after the implantation of the irradiated tumour grafts. The results were clear cut : the implanted viable grafts produced tumours in the hosts after a latent period of 10 to 16 days and the tumours grew steadily like those of the control mice. Because of the time lapse between the first and seconds graftings, further experiments were set up in an attempt to detect the possible existence of an early " immunity " of short duration, as follows :

(1) Tumour fragments which had been irradiated with 5300 r were implanted into 30 C3H mice (20 males and 10 females).

(2) Tumour fragments irradiated with 5400 r were implanted into 30 C3H mice (25 males and 5 females).

(3) Tumour fragments irradiated with 5500 r were implanted into 28 mice (20 males and 8 females)

EXPLANATION OF PLATES.

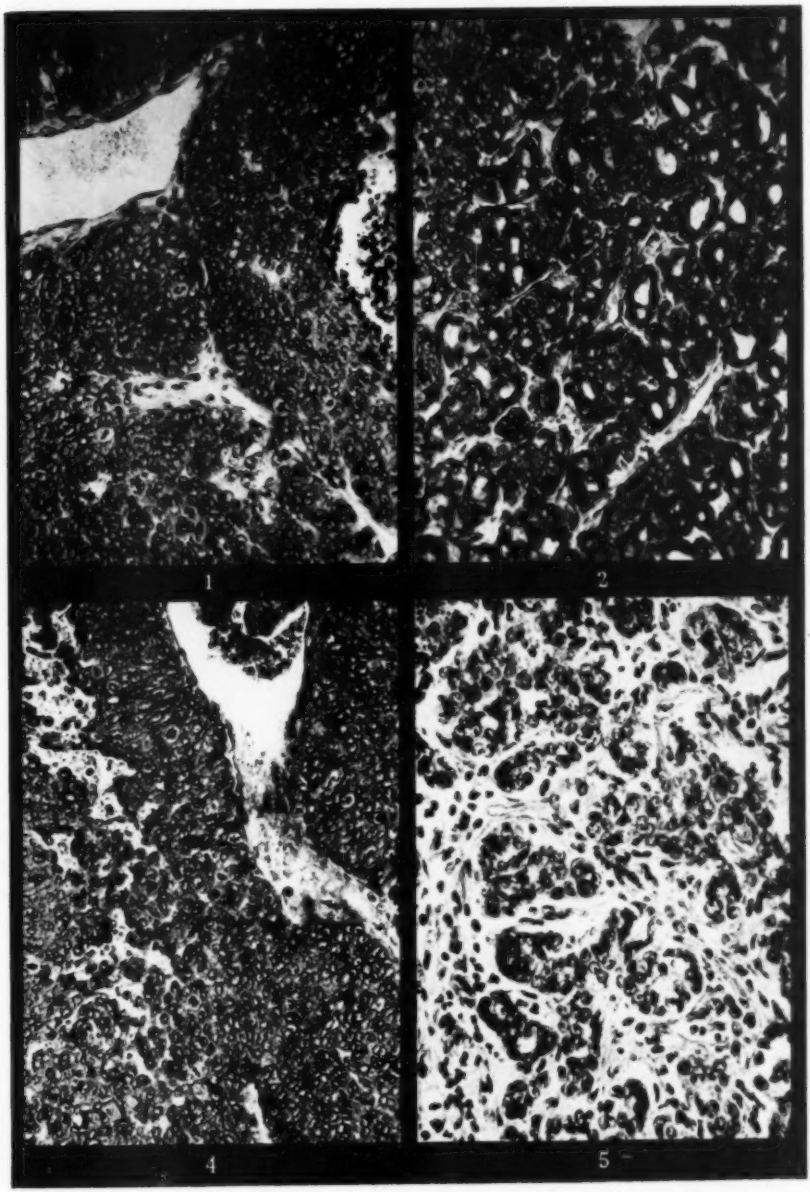
FIG. 1.—Primary mammary tumour which arose in a C3H mouse of the high cancer line (Bittner line) and designated C3HB. Sheets and bands of tumour cells closely packed. Some blood vessels, mitoses and necrotic areas are also seen. Mammary adenocarcinoma Type B, H and E. × 230.

FIG. 2.—Primary mammary tumour which arose in a DBA mouse of a high cancer line and designated DBAH. Acinar structures uniformly distributed in the parenchyma and separated by delicate stroma. Mammary adenocarcinoma Type A. H. and E. × 230.

FIG. 4.—Section of C3HB tumour removed 22 days following irradiation with 6000 r *in vivo*. Sheets and bands of tumour cells are seen, among which are mitotic figures, particularly in the areas near the blood vessel. Necrotic areas are also seen, but there is no fibrosis. H. and E. × 230.

FIG. 5.—Section of DBAH tumour removed 22 days following irradiation with 6000 r *in vivo*. Diffused fibrosis overgrowing the acinar structures can be seen. This is in contrast to Fig. 4. H. and E. × 260.

FIG. 6.—DBA mice in which the DBAH tumour regressed following irradiation with 10,000 r *in vivo*. Picture taken 18½ months after treatment.



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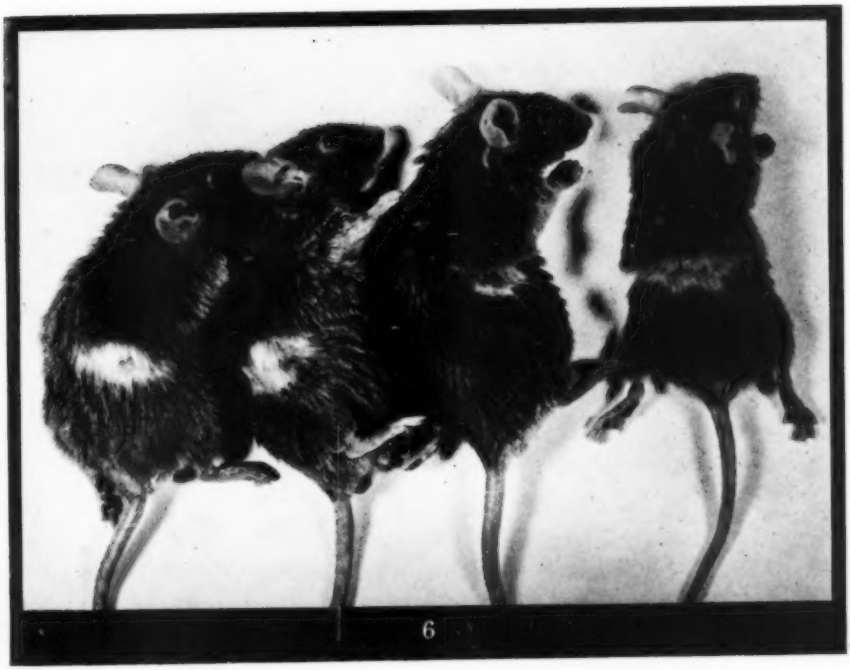

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(4) Fifteen C3H male mice were injected intradermally with tumour cells previously irradiated with 5400 r.

These doses of irradiation were used because, as shown in Table I, they constituted the attenuating dose*, and also because an overdose of radiation might destroy the immunizing ability of the tumour grafts (Wedd, Morson and Russ, 1914; Goldfeder, 1942).

TABLE I.—*Results of Irradiation of C3HB Tumour Grafts* in vitro.

Experiment number.	Dose (r).	Number of mice implanted.	Time of tumour appearance (days).	Number of mice with tumours.	" Takes " (%)
1	500	20	12–16	20	100·0
2	1000	20	13–18	20	100·0
3	1500	25	14–19	25	100·0
4	2000	25	16–23	25	100·0
5	2500	25	16–31	25	100·0
6	3000	25	23–31	25	100·0
7	3500	25	22–38	23	92·0
8	4000	25	24–58	19	76·0
9	4500	28	27–79	16	57·1
10	4700	22	28–84	10	45·5
11	5000	28	28–117	8	28·6
12	5300	24	28–73	3	12·5
13	5400	25	68	1	4·0
14	5500	25	—	0	0

None of the mice of the four series of experiments developed tumours within 2 weeks. They were then implanted with fresh, viable tumour grafts on the side opposite to that of the previous implantation. Ten mice in each series were grafted on both sides (left and right), in order to detect whether a local resistance was induced by the irradiated tumour fragments previously grafted on the right side. Table II gives a summary of the results. It can be noted that the fresh, viable tumour grafts produced palpable tumours in all mice used in the four experiments, following a latent period of 6 to 18 days. All the tumours grew progressively.

TABLE II.—*Results of Re-implantation of Viable Tumour Tissue into C3H Mice in which Irradiated Tumour Implants Failed to Grow.*

Number of experiment.	Number of mice.	Dose (r).	Number of " Takes."	Number of " Takes " on re-implantation.	Latent period (days).	Remarks.
1	30	5300	0	30	10–16	20 males and 10 females.
2	30	5400	0	30	10–16	25 males and 5 females.
3	28	5500	0	28	8–16	20 males and 8 females.
4	15	5400	0	15	6–18	All 15 mice were males. They were injected intradermally with irradiated tumour cells, but re-inoculated by the use of the trocar technique with viable tumour fragments.

* Definition of "attenuating" dose: A dose of irradiation which, when applied to tumour fragments *in vitro*, prevents their growth *in vivo* (after transplantation into the mice) but does not prevent their proliferation *in vitro* (in tissue culture) (Goldfeder, 1941, 1942).

Results obtained with C3HB transplantable mammary tumours treated in vivo.

A total of 189 C3H mice (148 males and 41 females) bearing the C3HB transplanted tumour were used in these experiments, and the results are recorded in Table III. Doses of 4000, 5000, and 6000 r produced no regression of the tumours. Retardation of growth and decrease in size of the majority of the treated tumours was noted within 10 days after irradiation, but later the tumours started to increase in size and they continued to grow. Microscopic examination of tumours removed 22 days after exposure to 6000 r showed mitotic figures (Fig. 4). All tumours, which had been treated with 7000, 8000, and 10,000 r respectively, decreased considerably in size within 10 to 14 days after exposure. However, only 20 per cent of the tumours treated with 7000 r, approximately 49 per cent of the tumours treated with 8000 r, and about 77 per cent of the tumours treated with 10,000 r regressed after the treatment. A dose of 12,000 r completely destroyed all the treated tumours. Sections of the tumours removed within 10 to 12 days after irradiation showed only necrotic tissue. It appears, therefore, that a dose within the range of 11,000 r to 12,000 r is lethal for the C3HB tumour of 1·0 to 1·5 cm. in diameter when grown *in vivo*. This dose is about twice as large as that necessary for attenuation of the tumour grafts, prior to implantation (Table I). The irradiated tumours which resumed their growth after initial decrease in size, grew steadily and in no instance did a tumour regress once it began to grow again.

Occasionally secondary tumours developed in the treated mice either in the inguinal or axillary regions within 2 or 3 weeks after the regression of the irradiated tumours. In some instances, new growth developed on the periphery of the irradiated tumour, probably from tumour cells which had not been included in the exposed area. The histological appearance of these secondary tumours resembled that of the primary, non-irradiated C3HB tumour.

Epilation and regrowth of gray hair appeared in the exposed areas 3 to 6 weeks after regression of the irradiated tumours. No ulceration of the skin in the irradiated area was ever noted in the C3H mice once gray hair appeared. A significant number of C3H mice lived about 1 year after regression of the tumours and spontaneous tumours appeared in some of the C3H females. Upon microscopic examination, these spontaneous tumours were found to be adenocarcinomata of the type usually occurring in mice of this C3H line.

B. *Observations on DBAH Transplantable Mammary Tumour.*

This tumour had been carried for 8 generations of serial transplants in the DBA mice from which it originated before it was used for irradiation. Although 100 per cent " takes " resulted in most instances, occasionally, however, an implant failed to produce a tumour in a DBA mouse which later proved to be resistant to further viable tumour grafts. According to Dr. Hauschka, this may be the result of a mutant histocompatibility factor present in the DBA strain ; in other words, an outcome of genetic incompatibility between the host and the transplanted tumour. To secure a better statistical evaluation of the results, a number of mice were grafted with viable, non-irradiated DBAH tumour fragments to serve as controls to those grafted with irradiated DBAH tumour implants. The results are recorded in Table IV. The first apparent effect of radiation on the tumour implants occurred after a dose of 1500 r. Two out of 28 mice failed to develop

TABLE III.—*Results of X-Radiation of C3HB Tumours* (1·0–1·5 *cm. in diameter*) in vivo.

Experiment No.	Number of mice.	Dose (r.).	Mice with regressed tumour (%).	Remarks.
1	12 males	4000	0	There was retardation of growth of the tumours during the first week after exposure; later the tumours increased in size and grew steadily.
2	15 „	5000	0	Six tumours decreased in size and nine retained their size during the first 10 days after irradiation but later all resumed their growth.
3	15 „	6000	0	All treated tumours decreased in size during the first week following exposure but later resumed their growth. Sections of the tumours taken 22 days after exposure showed active mitotic figures. (Fig. 4.)
4	15 „	7000	20·0	All 15 treated tumours decreased in size considerably during 10–14 days after irradiation. Three tumours regressed within 5 weeks after exposure. The skin healed and grey hair appeared on the irradiated site. The mice lived in apparent good health for 6–8 months. Eleven tumours resumed growth and grew steadily. Three tumours broke through the skin and ulcerated. Histological sections showed mitotic activity in mouse tumours.
5	33 (19 males, 14 females)	8000	48·5	In 16 mice (10 males and 6 females) the tumours regressed completely within 26 to 45 days after irradiation. The treated mice lived without tumours 6–10 months. Two females developed spontaneous tumours. Six tumours decreased considerably in size and later started to grow again and grew steadily. Eleven tumours decreased in size, leaving only small, yellowish nodes, but secondary tumours developed either in the inguinal or axillary regions. Microscopic sections of these tumours resembled those of the non-irradiated controls.
6	81 (56 males, 25 females)	10,000	76·54	The tumours in 44 males and 18 females regressed within 3 to 6 weeks after radiation. These mice lived for 6 to 10 months. Six of the young females produced litters. Four female mice developed spontaneous tumours. The tumours in the remaining 19 treated mice decreased to small nodes, but secondary tumours developed either in the inguinal or axillary regions.
7	18 (16 males, 2 females)	12,000	100·0	Fifteen of the treated mice (14 males and 1 female) were free of tumours during 3 to 5 weeks following X-ray treatment. Three (2 males and 1 female) of the treated mice died within 19 days after exposure of the tumours to X-radiation. The tumours in these mice had decreased to tiny nodes. Microscopic sections of these tumours showed only disintegrated tumour tissue. The males lived from 6 to 11 months after regression of the tumours.

tumours and they later proved to be " immune " to the DBAH tumour. The delay in the latent period is probably a more reliable criterion than the tumour " take " for re-evaluation of the radiation effects in this case, because it could be argued that the 2 mice in which the irradiated tumour cells failed to produce tumours possessed the mutant histocompatibility factor. In one experiment, a dose of 4500 r resulted in 22·9 per cent of "takes", while a dose of 4800 r resulted in 13·0 per cent of " takes " and a dose of 5000 r gave only 5·1 per cent of " takes ". However, in two additional experiments, after doses of 5000 r and 5200 r no "takes " occurred. The dose of 5000 r may, therefore, be regarded as the attenuating dose for the DBAH tumour implants. The radiosensitivity of this tumour is graphically presented in Fig. 3.

TABLE IV.—*Results of Experiments with DBAH Tumour Implants Control and X-Radiated* in vitro.

Dose (r).	Number of mice grafted.	Time of tumour appearance (days).	Number of mice with tumours.	" Takes " (%)	Number of mice without tumours.	Number of " Takes " after re-implantation.	Number of resistant mice.
Control	12	12–18	12	100·0	0	0	0
500	20	12–17	20	100·0	0	–	0
Control	10	14–16	9	90·0	1	0	1
1000	20	14–22	20	100·0	0	–	0
Control	20	12–17	20	100·0	0	–	0
1500	28	16–23	26	92·8	2	0	2
Control	25	14–18	24	96·0	1	0	1
2000	25	15–24	22	88·0	3	2	1
Control	25	13–18	24	96·0	1	0	1
2500	25	16–25	21	84·0	4	4	0
Control	25	12–18	25	100·0	0	–	0
3000	30	18–25	23	76·6	7	5	2
Control	25	14–16	24	96·0	1	0	1
3500	28	21–39	16	57·1	12	10	2
Control	20	14–18	20	100·0	0	–	0
4000	30	28–52	11	36·7	19	17	2
Control	25	15–17	23	92·0	2	0	2
4500	35	33–82	8	22·9	27	25	2
Control	20	15–18	20	100·0	0	–	0
4800	30	46	4	13·0	26	23	3
Control	20	14–17	19	95·0	1	0	1
5000	35	38	2	5·1	33	29	4
5000	25	—	0	0	25	24	1

Search for induced resistance.

Analysis of the data recorded in Table IV reveals that 7 out of a total of 227 control mice (about 3 per cent) were found to be refractory. None of these 7 produced tumours following implantations of viable tumour grafts. In the experimental mice, the number which failed to develop tumours was 150 out of a total of 341 mice which had been implanted with irradiated tumour grafts. When these mice were re-implanted with viable, non-irradiated tumour grafts, only 19 (about 6 per cent) remained resistant to re-inoculated DBAH tumour grafts. This difference is not statistically significant because of the larger number of the experimental mice.

The intradermal route was also used to test the possibility of inducing " immunity " in the DBA mice. Fifteen males and 10 females about 3 months old were injected intradermally, using the technique previously described, with a suspension

of the DBAH tumour cells which had been irradiated with 5000 r. None of these 25 mice developed tumours. Eighteen days later these mice were reimplanted subcutaneously with fresh, viable tumour grafts. Palpable tumours, which grew progressively, appeared within 9 to 13 days in 14 out of the 15 reimplanted male and in 9 out of 10 re-implanted female mice.

Two mice, one male and one female, in which the irradiated tumour cells failed to produce tumours in the first place, remained refractory to twice repeated implantations of viable tumour grafts. This result may also be ascribed to the mutant histocompatibility factor present in these 2 mice.

Results of X-radiation of DBAH tumours in vivo.

Table V records the observations made on a total of 227 mice (164 males and 63 females). As can be noted, doses of 4000 and 5000 r produced, in no instances, a total regression of a tumour. A significant number of tumours, however, decreased to small firm nodules which, after 2 months or longer, resumed growth. Microscopic sections taken from the regressing tumours showed an increased proliferation of connective tissue (Fig. 5) when compared with non-irradiated DBAH tumours of the same generation of transfer. This is in contrast to the C3HB tumour shown in Fig. 4. A rather sudden increase in the number of tumours which showed regression was noted following the application of doses higher than 6000 r. Thus, 60 per cent of the tumours regressed after a dose of 7000 r, while a dose of 6000 r destroyed only 25 per cent of the treated tumours. The larger percentage of the regressed tumours which had been treated with a dose of 10,000 r indicates that this is the destructive dose for the DBAH transplantable tumours, ranging in size from 1·0 to 1·5 cm. in diameter. As in the case of the C3HB tumour, this dose is twice as large as the dose necessary for the attenuation of grafts of this tumour *in vitro*.

The majority of the mice in which tumours had regressed lived in apparent good health for 1 year or longer, and the young female mice produced apparently normal litters. As in the C3H mice, spontaneous tumours appeared in the DBA females after regression of the treated transplantable tumours, and gray hair appeared on the site of exposure 2 to 3 weeks after the regression of the tumour in the irradiated area. Only in two instances did ulceration of the skin occur. In one instance the ulceration occurred 11 months and in the other 16 months after disappearance of the tumour. The ulcerations persisted until the death of the animals. These two instances may parallel radiation necrosis occasionally noted in human patients treated with X-rays. The mice which survived 18½ months after regression of the tumours are shown in Fig. 6.

DISCUSSION.

Ever since Ehrlich (1906) made his classical observation that an animal host became resistant or " immune " to re-implanted grafts of the same tumour after a tumour graft failed to " take", a widespread interest was shown in the theoretical and practical implications of this phenomenon. Many attempts were made to induce immunity to cancerous growths. Haaland (1909); Contamin (1910); Mottram and Russ (1917); Chambers, Scott and Russ, (1922); Clemmesen, (1938) have shown that a resistant state can be induced by implanting the animals with tumour grafts previously attenuated with ionizing radiation. However, it

TABLE V.—*Results of X-Radiation of DBAH Tumours, (1·0–1·5 cm. in diameter) in vivo.*

Number of experiment.	Number of mice.	Dose (r).	Cured mice (%)	Remarks.
1	15 males	4000	0	Eight of the treated tumours decreased in size after irradiation but resumed their growth and grew steadily. The tumours in the remaining 7 mice grew steadily.
2	20 (15 males, 5 females)	5000	0	Fourteen tumours decreased considerably in size within 10 days after irradiation but later increased in size. Six tumours grew steadily after exposure.
3	20 (16 males, 4 females)	6000	25	Five tumours regressed within 3 to 4 weeks following exposure. The cured mice lived normally for 8–10 months after treatment. Four treated tumours shrank to small, firm nodes but metastatic tumours developed in distant regions. Eleven tumours continued to grow after exposure. The firm regressing tumours contained considerable amounts of fibrous tissue.
4	25 (17 males, 8 females)	7000	60·0	Tumours in 6 females and in 9 males regressed within 14–28 days after irradiation. Seven tumours decreased in size but resumed their growth later. Three mice died within 10 days after treatment. The tumours in these mice had decreased in size. Microscopic sections showed extensive necrosis in the centre. but the edges contained intact cells and mitoses. Two females developed spontaneous tumours 6 months after regression of the treated tumours.
5	35	8000	77·14	In 27 mice (20 males and 7 females) the tumours regressed completely within 3 weeks after irradiation. The cured mice lived normally thereafter, some up to 1 year and longer. Four females developed spontaneous tumours. In the remaining 12 treated mice the tumours decreased considerably in size. In 5 of these the tumours resumed their growth and in 7 the tumours decreased to small, firm nodes while simultaneously metastatic tumours developed in distant regions. Microscopic sections of the regressing tumours consisted mainly of fibrous tissue.
6	35 (21 males, 14 females)	9000	85·7	The tumours regressed completely in 30 treated mice (21 males and 9 females). Five females developed spontaneous tumours 6–7 months after regression of the treated tumours. The treated mice lived for as long as 1 year and more after treatment. In the remaining 5 mice, the tumours decreased in size to small, firm nodules, but metastatic tumours developed in regions distant from the exposed areas. Microscopic sections of the regressing tumour nodules consisted of necrotic and fibrous tissue.

TABLE V—*cont.*

Number of experiment.	Number of mice.	Dose (r).	Cured mice (%)	Remarks.
7	78 (59 males, 19 females)	10,000	93·6	In 57 males and 16 females the tumours regressed completely within 3–6 weeks. One female mouse died 8 days after irradiation of the tumour and another female mouse developed a metastatic tumour in the inguinal region. The 2 remaining male mice developed metastatic tumours on the edges of the exposed areas. The young females produced litters. Seven of the older treated females developed spontaneous tumours. The mice lived in apparent good health up to 1 year and more. Of all treated mice only in two instances did ulceration of the skin in the irradiated area occur 11 months after regression of the tumour.

should be noted that in the experiments cited above, genetically impure animals and tumours genetically different from the hosts were used. The writer's interest in the problem of induced resistance arose from investigations on mouse Sarcoma 180 grown in white mice of the Swiss strain (Goldfeder, 1942). The mice were made resistant to this tumour in almost 100 per cent of the cases following implantation with irradiated Sarcoma 180 grafts. The writer explored the possibility of inducing a resistant state in inbred animals by implanting into these animals irradiated grafts of a tumour which originally developed in an animal of the same inbred line. These experiments were carried out in an attempt to ascertain whether ionizing radiation induces genetic and antigenic changes in the tumour cells which make them foreign to the host in which they originated. Experiments were also carried out on a reticulum cell lymphosarcoma, using Bagg's strain of rats (Goldfeder, 1945*b*). According to the data given by the late Dr. Bagg, the tumour had arisen in a rat of this particular line which had been inbred for 15 years. Tumour grafts exposed to doses ranging from 2200 to 2800 r failed to grow after implantation into the normal rats. These rats later proved to be resistant to re-inoculated viable tumour grafts. In the light of the present experiments it appears that this was due to the fact that the rats of this line were not entirely inbred. This is supported by the observation made during the experiments on the control, non-irradiated tumour grafts : On occasions when the non-irradiated tumour graft failed to " take," that rat became refractory to re-implanted viable tumour grafts.

The failure to induce resistance in inbred strains of rats to transplantable autogenous tumours was brought forth in more recent experiments. Thus, grafts of the mammary adenocarcinoma autogenous to the August inbred strain of rats, as well as grafts of the fibrosarcoma autogenous to the A–C rats attenuated with critical doses of X-rays, failed to render the rats resistant to re-implanted viable tumour grafts of the respective tumours (Goldfeder, 1953).

In the present experiments control, non-irradiated DBAH tumour grafts failed to grow in about 3 per cent of mice of the DBA strain in which the tumour originated. These mice were found to be resistant to re-implantation of viable grafts of the same tumour. Among the DBA mice in which the irradiated DBAH tumour grafts failed to take, 5·7 per cent proved to be resistant to re-inoculated,

viable tumour grafts. This small difference in percentage is not statistically significant because of the larger number of mice used for the experiments on irradiation. These resistant DBA mice were taken to have a different genetic constitution from that of the DBAH tumour. These observations, combined with those made on inbred (homozygous) C3H mice and the autogenous mammary adenocarcinoma designated C3HB, brought forth the importance of using genetically pure and identical material (tumour and host) in this type of experiment in order to secure reliable results. The failure to induce resistance in hosts by irradiated autogenous tumour grafts emphasizes the strong genetic relationship between the tumour cell and the host. The irradiation of tumour cells in the present experiments did not make them foreign or antigenic to the host in which they originally developed.

An analysis of the results recorded in Table I and in Table III reveals a difference in the radiosensitivity between the C3HB and DBAH tumours. For example, a dose of 3500 r gave 92 per cent " takes " of the C3HB tumour grafts and only 57·1 per cent " takes " of the DBAH tumour grafts. At the borderline of the attenuating dose, the difference in the radiosensitivity of these tumours is only about 500 r. This range of attenuation dosage for both tumours is in line with that determined for implants of other mammary adenocarcinomas in inbred strains of mice (Lawrence, Horn, Strong, 1937 ; Goldfeder, 1945*a*) and for a mammary adenocarcinoma of an inbred line of rats (Eisen, 1940). On the other hand, the C3HB mammary adenocarcinoma used in the present studies proved to be more resistant to irradiation than the C3H mammary adenocarcinoma used in previous experiments (Goldfeder, 1947), the latter requiring only 2700 r to prevent its grafts from " takes ". The difference in the radiosensitivity between the C3HB and C3H mammary adenocarcinomas may be explained by (*a*) a difference in degree of differentiation : the C3HB mammary adenocarcinoma in the present experiments is a more anaplastic type of tumour consisting of sheets and bands of closely packed cells, with relatively little fibrous connective tissue, while the C3H mammary adenocarcinoma previously reported (Goldfeder 1947) consisted of acini, uniformly distributed and separated by connective tissue ; (*b*) by a difference in genetic make-up between the tumours and their hosts : the C3HB mammary tumour was carried by serial transplants in pure inbred C3H mice from which it originated, while the mammary tumour, used in previous studies (1947–1950), was carried in C3H mice of different sublines and thus the tumour and the hosts were not of the same genetic origin. Failure of the tumour to " take " in about 10 per cent of the hosts indicated that some of the C3H mice used in the experiments of 1947–1950 had a different genetic constitution than that of the mouse in which the tumour developed. The skewed curve illustrated in that publication (Goldfeder, 1947) in contrast to the typical S-shaped curve obtained in the present experiment may also be the result of the difference in the genetic make-up between the hosts and the tumour. The radiosensitivity of tumours depends to a large extent on whether they are growing in hosts of the same strain from which they originated, or in foreign hosts as shown by Aughterson, Tennant, and Lawrence (1940). Irradiation of a transplantable mammary carcinoma in Strain A mice in which the tumour originated induced regression only in 1 per cent of the treated mice when 2500 r were applied and in 48 per cent of mice following the application of 5000 r. When these doses were applied to the same tumour grown in hybrid mice, tumour regression was induced in 82 per cent

and 97 per cent of cases respectively. Thus tumours growing in animals of a strain in which they originated required significantly larger doses of radiation than those growing in foreign hosts. The antigenic reaction of the foreign host is thus a contributing factor in rendering the tumour more vulnerable to regression after irradiation.

The more anaplastic C3HB tumour proved to be more resistant to radiation than the relatively more differentiated DBAH tumour. This corroborates the observations made by the writer on another mouse mammary adenocarcinoma (Goldfeder, 1950*a*) and those noted on human tumours. For example, Cathie (1939), Lenz (1942) and Glücksmann (1948) found that well differentiated cancers regressed more readily and offered a better prognosis than the less differentiated tumours.

The rôle of connective tissue in the response of animal tumours to irradiation and its relationship to the host was brought forth by Cramer (1932) and by Aughterson, Tennant and Lawrence (1940). The relatively more pronounced proliferation of the connective tissue in the DBAH tumour than in the C3HB tumour may also be regarded as a contributing factor rendering the DBAH tumour more vulnerable to irradiation. The observation of secondary tumours occurring in the treated mice also seems to parallel observations made on human mammary cancers. This observation indicates the failure of the disintegrated irradiated tumour tissue to induce resistance in the hosts to new cancerous growths.

It has been noted that untreated metastatic tumours often regress after X-ray treatment of primary tumours (Sugiura, 1937 ; Aughterson, Tennant and Lawrence, 1940). This observation has been attributed to a supposed indirect effect of irradiation (Sugiura, 1937). These observations could probably be explained by the fact that the tumours were grown in genetically foreign mice. The present experiments on mice of genetically pure strains and their autogenous tumours indicate that the indirect effect of irradiation is not sufficient to affect secondary growths. Regression of the secondary tumours in X-ray treated heterozygous mice may be attributed to an antigenic effect, elicited by the genetically differing tumour tissue. The inability of the irradiated tumour tissue to induce " immunity " in hosts from which it originated may indicate that no qualitative change of a genetic or antigenic character occurred in the irradiated tumour cells which would render them foreign to the hosts. Inbred lines of mice and their tumours offer far more suitable material for investigations of this type than heterozygous mice. The observations made from such studies seem to parallel those noted on human mammary cancers.

In earlier publications (Goldfeder 1950*b*, 1952), the writer showed that a fast growing, less differentiated mouse mammary adenocarcinoma also possessed a more vigorous metabolic rate and suggested that this property may have been one of the factors influencing the radiosensitivity of the mammary adenocarcinomas then used. Whether this also holds true for the C3HB and DBAH mammary adenocarcinomas now described is as yet not known. Experiments on the metabolic behavior of these tumours are in progress.

SUMMARY.

The radiosensitivity and ability to induce a resistant state in mice of the same strain were studied in two transplantable mammary adenocarcinomata, designated

C3HB and DBAH, which had originated in mice of the respective inbred C3H and DBA high cancer strains.

A difference in the radiosensitivity, both *in vivo* and *in vitro*, was observed between C3HB and DBAH transplantable mammary tumours. A smaller irradiation dose was found to prevent the irradiated DBAH tumour grafts from growing *in vivo* than was required to produce a similar effect on C3HB tumour grafts. Similarly, for treatment of established tumours *in vivo*, the dose of irradiation which produced regression of the DBAH tumour was found to be smaller than that which had a similar effect on the C3HB tumour. Some factors which are involved in the difference in the radiosensitivity of these two tumours are the state of differentiation of the tumour cells and the amount of stroma.

In both instances about half of the radiation dose, producing destruction of the established tumours grown in their respective hosts, was required to obtain the attenuation of the grafts of both C3HB and DBAH tumours.

The C3H mice in which the irradiated *in vitro* C3HB tumour grafts failed to " take " or in which a well developed transplanted C3HB tumour regressed after irradiation *in vivo* remained susceptible to reimplanted viable C3HB tumour grafts. A small number of the DBA mice in which the viable DBAH tumour grafts failed to " take " *in vivo* remained resistant to viable tumour grafts. Similarly, a small number of the DBA mice in which the irradiated *in vitro* tumour grafts failed to " take " was found resistant to re-implantation of fresh viable tumour implants. This is explained by a difference between the genetic make-up of some of the DBA mice and the DBAH tumour.

A significant number of mice of both strains lived in apparent good health for 1 year and longer after regression of the *in vivo* irradiated tumours ; among these survivors the young females produced apparently normal litters. Spontaneous tumours also occurred among these female survivors on reaching cancer age.

Thus, under the experimental conditions described, the question about the possibility of inducing immunity in homozygous hosts to endogenous tumours by irradiation of these tumours appears to have been answered in the negative. The part played by the genetic relationship between the tumour and host in the induction of resistance to the appearance of tumours was brought forth. The importance is stressed of using tumour material genetically identical with the host in irradiation experiments.

My sincere thanks are due to Dr. J. J. Bittner for the C3H high cancer line mice and to Dr. T.C. Hauschka for mice of the DBA high cancer line which they so kindly supplied for breeding purposes. Grateful acknowledgment is made to Carl B. Braestrup, Senior Physicist of the Department of Hospitals, New York City, and his associates for supplying physical measurements ; to F. Inouye for excellent laboratory assistance and co-operation.

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THE DEVELOPMENT OF RESISTANCE IN THE WALKER CARCINOSARCOMA TO THE ACTION OF TRIETHYLENE MELAMINE.

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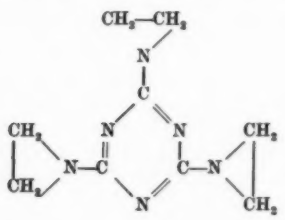
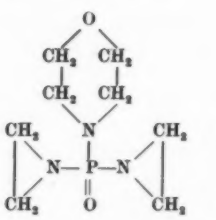
THE development of resistance to chemotherapeutic agents is a familiar phenomenon in the investigation and treatment of most diseases of infective origin. It is probable that the production of a refractory state is, as yet, an invariable result in the chemical treatment of those few cases of malignant disease which respond satisfactorily in the first instance.

The appearance of resistance to specific chemical inhibitors has been recognised and investigated in some kinds of mouse leukaemia (Burchenal, Robinson, Johnston and Kushida, 1950; Law, 1952*a*, 1952*b*) but so far it appears that no convincing demonstration of this important phenomenon has been made in solid animal tumours.

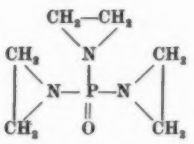
This communication records the development of a persistent refractory state in the Walker carcinosarcoma after treatment with triethylene melamine (I), to which substance the tumour is normally very sensitive; the resistant state is also shown to extend to two related substances, II and III.



I.
2 : 4 : 6-Tris-ethyleneimino-1 : 3 : 5-triazine
(Triethylene melamine)
"T.E.M."



II.



III.
N^1, N^2, N^3-tris-ethylenephosphoramide
(T.E.P.A.).

MATERIALS AND METHODS.

Animals.—Rats of an American Wistar strain were used, maintained on a cube diet supplied by the North Eastern Agriculture Society. During experiments each animal was allowed 15 g. daily.

Tumour.—The Walker carcinosarcoma was originally obtained from the Biological Laboratories of Imperial Chemical Industries. It was easily adapted to these animals and was maintained by subcutaneous dorsal transplantation into young adult rats (110-140 g.).

Assessment of chemotherapeutic potency.—Usually groups of ten animals (equal numbers of each sex) were used as controls and similar groups were given daily doses of the drug by the intraperitoneal route, with a double dose on Saturday. Except where stated, dosage was commenced 4–5 days after transplantation. The drug was dissolved in water and kept in the refrigerator, the solution being replaced every few days. Daily inspection of the tumour site was made and the subsequent growth of the neoplasm estimated by palpation using an arbitrary scale of measurement of its size in relation to the bulk of the animal. The animals were killed 10–14 days after implantation and the tumours dissected out and weighed. The decision to terminate an experiment was governed by the rate of growth of the tumour in the control series of animals, which varied somewhat in different experiments. The effect of treatment was measured by comparing the mean tumour weights in control and treated groups and use was also made of the formula suggested by Walpole (1951), which makes allowance for those animals offering undue resistance to the growth of the tumour.

RESULTS.

Fig. 1 represents the collected results of the main series of experiments. In the first of these, three groups of ten animals were transplanted with the Walker tumour. Ten were used as controls, ten received triethylene melamine, 0·1 mg./kg. daily, and the remainder received twice this dose. Table I shows the result of this experiment on the group receiving the lower dose level; the treated tumours were completely inhibited. The ten animals receiving the higher dose level were not killed, but drug treatment was discontinued after eight consecutive daily doses. Small nodules persisted in the majority of these animals without change for about 2 weeks, after which tumours began to grow; they were well developed in 70 per cent of the group 1 month after cessation of treatment. One

TABLE I.—*Inhibitory Effect of Triethylenemelamine* (0·1 *mg./kg. daily, i.p.*) *on the Walker Carcinosarcoma.*

	Controls.	Treated.
Number of animals	10	10
Treatment	None	8 doses commencing 2nd day
Day terminated	12	12
Number surviving	10	10
Tumour weights (g.)	17 8	0·5 0·2
	12 4	0·5 0·2
	9 0	0·4 0·1
	9 0	0·3 0·0
	9 0	0·2 0·0

of the tumours was selected at random, transplanted as before, and subjected to a second course of treatment (Fig. 1). This time, growth occurred in spite of administration of the drug and after 13 days the experiment was terminated. A subsidiary transplant was made from one of the *treated* tumours and a group of ten animals carrying the neoplasm again exposed to the action of the compound. The development of tumours was not markedly depressed compared with the control series and in the next experiment one of these *treated* tumours was once more subjected to a repetition of the above procedure (Fig. 1, fourth treatment). From this time (Fig. 1, " 32 weeks ") one of these tumours (the *derived* tumour)

has been maintained routinely by transplantation at intervals of about 2 weeks—in all about 50 such transferences have since been carried out. From time to time the sensitivity of the tumour to triethylene melamine was tested (Fig. 1) The continued refractory behaviour of the derived tumour compared with the original Walker tumour is clearly evident. This resistance to further treatment shown by such tumours has been confirmed. Thus in another experiment a

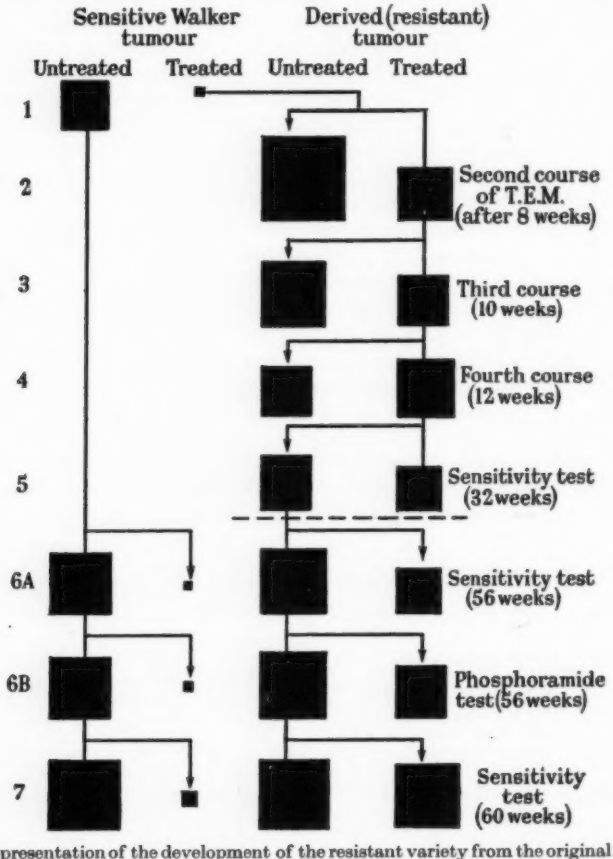


FIG. 1.—A representation of the development of the resistant variety from the original sensitive Walker carcinosarcoma, following treatment with triethylene melamin (T.E.M.). The black squares are drawn to scale and represent the mean weight of tumours at the end of an experiment. Experiment 1 shows the effect of the drug (0·1 mg./kg.) on the Walker tumour. The inhibited tumours later grow, and Experiments 2, 3, 4 and 5 show that repeated exposure to the drug has little effect on the growth of the derived tumour; also that there appears to be no increase in resistance with successive treatments. After Experiment 5, the derived tumour was maintained by transplantation, with occasional tests for susceptibility to the ethyleneimine (Experiments 6A and 7). Experiment 6B represents the result of a test using the diethylenephosphoramide (II) referred to in the text. The control series in the left hand column illustrate the continued susceptibility of the original Walker tumour. In between the experiments referred to above, the resistant tumour was maintained by transplantation about every 10th day.

similar tumour (after six intermediate transplants) was tested for sensitivity to triethylene melamine. The treated animals received 6 daily doses of the drug (0·2 mg./kg., commencing the 3rd day after transplantation) and the experiment was terminated on the 12th day.

Untreated animals.—Tumour weights (g.) 30, 25, 15, 15, 11.

Treated animals.—Tumour weights (g.) 15, 14, 14, 13, 10, 9, 8, 4, 3, 2.

The resistance shown by this tumour was of similar magnitude to that shown by the tumour referred to in Fig. 1 and it seems reasonable to suppose that the same applies to all such tumours developing from inhibited nodules.

When drug treatment was commenced 3–5 days after transplantation, both the sensitive Walker tumour and its refractory derivative were always actively growing. The former invariably began to regress within 24–48 hr. of the first dose, whilst the latter continued to grow. When treatment with triethylene melamine was commenced 24 hr. after implantation of the tumour, complete inhibition of the original Walker tumour resulted as was expected. The comparative tumour weights from this experiment, terminated after 7 daily doses were :

Controls.—20, 16, 15, 14, 14, 13, 13, 12, 12, 6, (g.).

Treated.—The largest nodule was 300 mg., including a considerable proportion of necrotic tissue.

The nine animals remaining in the treated group were kept and tumours began to grow 2–3 weeks later. Five weeks from the date of termination of treatment the experiment was concluded and the tumour weights were as follows :

53, 25, 15, 15, 4, 3, 2, 0, 0 (g.).

The derived tumour, in a parallel experiment, was also treated with the same drug for the same time, commencing 24 hr. after implantation. It developed much as usual, although not at the same rate as in untreated controls :

Untreated animals.—Tumour weights (g.) 24, 22, 20, 20, 19, 19, 18, 10, 7.

Treated animals.—Tumour weights (g.) 15, 11, 10, 9, 7, 4, 4, 3, 1, 0·5.

Thus even early treatment before the tumour had established itself in the host animal, failed to eliminate those cells of the original Walker tumour which later develop into the refractory tumour. Such early treatment was also without effect on the growth of the resistant tumour, compared with delaying drug administration until the graft was well established (3–5 days).

By way of comparison, Table II illustrates the effect of triethylene melamine (0·2 mg./kg.) on the growth of a lymphosarcoma in the same strain of rat. Tumours grew well in treated animals although some inhibitory effect is apparent from the final data.

Table III shows the " inhibitory index " calculated for various experiments. Inhibition of the original Walker tumour by the three ethyleneimines referred to was always virtually complete—it is in fact greater than the results indicate, for the nodule always contained a variable amount of material other than tumour tissue. The growth of the derived tumour was affected to a reasonably consistent extent by triethylene melamine, comparable to that observed with the lymphosarcoma.

The subsequent development of inhibited Walker tumour nodules has been repeatedly confirmed. Surprisingly, growth was found to occur after a similar latent period in the majority of animals even though drug treatment was continued

TABLE II.—*Effect of Triethylene Melamine (0·2 mg./kg. daily, i.p.) on the Growth of a Lymphosarcoma.*

The degree of inhibition is comparable to that caused in the resistant form of the Walker tumour. How far this depression of growth is related to the systemic toxicity of the drug on the host animals is not known.

	Controls.		Treated.	
Number of animals	8		10	
Treatment	None		6 doses commencing 4th day	
Number surviving	8		10	
Day terminated	13		13	
Tumour weights (g.)	37	22	20	9
	32	2	13	9
	32	0	11	4
	27		10	1
	22		10	1

TABLE III.—*Inhibitory Effect of Triethylene Melamine (T.E.M.), Triethylene Phosphoramide (T.E.P.A.) and a Diethyleneimino-compound (Compound II) on the Growth of the Walker Carcinosarcoma and the derived (Resistant) form of this Tumour.*

The "inhibitory index" is based on the formula given by Walpole (1951), namely:

$$\text{Inhibitory Index} = \frac{(M_{50}\ \text{controls} - M_{50}\ \text{treated})}{(M_{50}\ \text{controls})} \times 100$$

where M_{50} refers to the n heaviest tumours out of any group of 2_n.

Tumour.	Drug.	Treatment (doses).	Day terminated.	Inhibitory index per (cent).
Original Walker	T.E.M.	8	12	98
" "	"	8	13	98
" "	"	5	13	95
" "	T.E.P.A.	6	11	96
" "	Compound II	8	13	99
Derived Walker	T.E.M.	8	13	55
" "	"	10	14	53
" "	"	9	14	34
" "	"	6	10	57
" "	"	8	13	57
" "	"	5	11	18
" "	T.E.P.A.	6	11	44
" "	Compound II	8	13	30

throughout at the same rate. An example of this is shown in Table IV, from which it is evident that tumours developed in 8–10 animals following the usual inhibitory period of nearly 3 weeks. Once obvious growth commenced, it proceeded rapidly and then slowed quite markedly. In spite of the prolonged treatment in which 31 doses were given, there were no deaths which could be directly attributed to the effect of the drug. This failure of continued treatment to maintain the state of inhibition in the tumour has also been confirmed in other experiments.

The contrast in susceptibility between the original and derived Walker tumours to the triazine, has also been found to extend to the two other ethyleneimines, II and III (Tables III and V). The resistant phenomenon seems therefore to be

TABLE IV.—*Effect of Continued Treatment of Rats bearing Implanted Walker Tumour with Triethylene Melamine* (0·2 *mg./kg.*).

Administration of the drug was started on the 4th day with the result that existing tumours regressed and remained inhibited. After a latent period of nearly 3 weeks, tumours began to grow in spite of continued exposure to the drug. In all, 31 doses of the compound were given. One animal was killed on Day 27, when it was found that the tumour had penetrated the abdominal wall and spread widely through the retroperitoneal space and mesenteries. The left kidney had been invaded and the pancreas partially destroyed. One animal died early on in the treatment.

Days after transplantation.	Number of animals showing: No tumour growth.	Nodule.	Growth. +	++	+++	++++
9		9	1			
13	1	8	1			
20	1	2	6			
23	1	1	1	6		
28	1	0	1	1	2	3
29	1	0	0	1	3	3
32	1	0	0	1	1	5
38	1	0	0	0	1	6
49	Experiment terminated ; tumours weighed.					
	Tumour weights (g.) :		40	18		
			34	11		
			25	10		
			19	0		

TABLE V.—*Two Experiments Showing the Effect of two Ethylene phosphoramides* (*Compound II and Triethylenephosphoramide*) *on the derived Walker Tumour.*

These substances produce a similar inhibitory effect on the original Walker carcinosarcoma to triethylene melamine (Fig. 1 and Table I), but have only a small retarding effect on the derived tumour.

	Control.	Treated.
Compound II (2·0 mg./kg.)—		
Number of animals	10	10
Treatment	None	8 doses commencing 4th day
Day terminated	13	13
Number surviving	10	10
Tumour weights (g.)	27 18	25 11
	23 16	24 7
	25 13	18 4
	21 5	16 4
	18 0	14 3
T.E.P.A. (2·0 mg./kg.)—		
Number of animals	10	10
Treatment	None	6 doses commencing 5th day
Day terminated	10	10
Number surviving	10	10
Tumour weights (g.)	39 20	22 11
	30 20	18 8
	23 19	13 3
	23 17	13 3
	21 6	12 0·5

related to the presence of reactive ethyleneimino-groups rather than to general molecular pattern.

DISCUSSION.

The conversion of a tumour normally sensitive to a drug into one which is refractory, is of particular interest in relation to the further study of the mode of action of chemotherapeutic agents; investigation of such phenomena may contribute to a better understanding of the behaviour of malignant tumours in man, and help to explain the general lack of correlation between human and experimental tumours in their response to chemical inhibitors. In the few cases of human malignant disease showing an initial favourable response to chemotherapy, the development of resistance seems so far, to be inevitable.

The results described in this paper appear to be the first account of the experimental production of resistance in a solid tumour in the rat, and the first of its kind in any species relating to this category of chemical inhibitor whose action is apparently related to its high chemical reactivity. As was mentioned earlier, the development of mouse leukaemia resistant to folic acid analogues is well known and there has been a recent report that the Mouse Sarcoma 180, which is moderately inhibited by 6-mercaptopurine, rapidly develops some resistance to this compound (Clarke, Philips, Sternberg, Stock, Elion and Hitchings, 1953).

On the whole, solid rat and mouse tumours appear to be relatively resistant to chemical inhibitors, although the response of different tumours to the same drug varies widely. Not often does the arrest of growth extend to all tumours in an adequate group of experimental animals, in spite of the administration of dose levels of near lethal magnitude (Suguira and Stock, 1952*a*, 1952*b*). It appears that rat tumours are more susceptible than those of the mouse to triethylene melamine, for complete regression has been obtained in nearly 100 per cent of animals with the Jensen rat sarcoma, Sarcoma R39 and the Flexner Jobling carcinoma (Suguira and Stock, 1952*b*). It is generally agreed that the longer initiation of treatment is delayed, the less chance there is of total regression. The action of triethylene melamine on the Walker carcinosarcoma is striking even when treatment is delayed for as long as 5 days after implantation, by which time the tumour is actively growing. In the present series of experiments regression occurred in every animal and usually little or no palpable material remained at the site of the implant. This confirms the work of Hendry, Homer, Rose and Walpole (1951) and Peczenik (1952), who however, commenced treatment 24 hr. after transplantation of the Walker tumour into Wistar rats. Curiously, Suguira and Stock (1952*b*) were less successful with this tumour in Wistar rats, obtaining inhibition of growth in 70 per cent of animals with day old implants and regression in only 20 per cent when treatment was delayed for 7 days. The complete inhibition of this tumour in our animals first suggested that a demonstration of the development of a resistant variety might be feasible. The results clearly show that in effectively all animals the inhibitory effect is only temporary and that growth subsequently occurred irrespective of the duration of treatment, even though this may be continued for several weeks instead of the usual 7–10 days. In either case the suppression of growth continued for upwards of 2 weeks before renewal of activity could be detected. When administration of drug began 24 hr. after implantation of the tumour and no growth occurred at all during the period for which it was given, tumours subsequently developed as

usual 2–3 weeks after discontinuation of treatment in 9/10 animals. The derived tumour on which most of the work has been carried out, was selected at random from a group of tumours growing subsequent to one short course of treatment applied to the original Walker tumour. The resistance it displayed has remained essentially unchanged by further exposure to the drug. The tumour has since been transmitted over 50 times without interposed treatment and tests have not revealed any increase in sensitivity during this time. The resistance is thus considered to be a stable and permanent change. Another member of a group of tumours similarly derived from the original Walker carcinosarcoma has also been shown to possess comparable resistance to further treatment. It is reasonable to suppose that the same will apply to all such tumours. A noticeable difference between the original Walker and the derived tumours is the more rapid growth of the latter in untreated animals.

The growth of tumours after a latent period following a short course of treatment with triethylene melamine, must be due to the survival of a small number of cells which are naturally resistant to the drug. The fact that this sequence of events is not changed by the continued administration of triethylene melamine to animals bearing implants of the original Walker tumour, suggests that the resistance of the residual cells is maximal at the outset. Additional support for this view emerges from the failure to enhance the resistance by further exposure to the drug. How far mutagenic effects of the ethyleneimine may be concerned remains to be investigated, but the fact that such a high proportion of inhibited tumours ultimately grow seems to make this factor less likely. The resistant tumour has also been shown to be refractory to two other ethyleneimines (Compounds II and III) at dose levels which completely inhibit the original Walker carcinosarcoma. Thus resistance to triethylene melamine confers resistance to a diethyleneimine, and this may be true of active ethyleneimines in general. How far the same statement may apply to other kinds of chemical inhibitors active against the ordinary Walker tumour is being investigated.

It is interesting that the Walker tumour should be so sensitive to triethylene melamine whilst two other tumours which have been tested in the same strain of animal (a mammary adenocarcinoma and the lymphosarcoma referred to in the text) were only little affected by the same treatment. Crossley, Allison, Wainio and Muenzen (1951) studied the effect of triethylene melamine on a sarcoma (231) in the King A rat. They reported that a dose of 0·1–0·2 mg./kg. daily produced severe toxic symptoms and no tumour regressions were obtained under these circumstances; 0·2 mg./kg. twice daily was lethal to these animals. With smaller doses, e.g., 0·025 mg./kg. they were able to obtain complete regression in from 50–75 per cent of animals. From their results it appears that tumours not inhibited by the drug treatment were able to grow at rates not very different from those in untreated animals. Crossley, Allison and Muenzen (1952) also reported the effect of the same drug on the Flexner-Jobling carcinoma in Sprague-Dawley rats, and found complete regression in 70 per cent of all treated animals with none in control series. Regressions were stated to occur with doses ranging from 0·016–0·046 mg./kg. twice daily, without evidence of toxic effects. Doses of 0·23 mg./kg./day in 4 divided doses caused regression in all animals treated not later than 8 days after transplantation. The cured animals showed no recurrence up to 1 year after treatment. The authors comment that this strain of rat was less sensitive to triethylene melamine than the King A variety.

Suguira and Stock (1952*b*) used 0·25 mg./kg. of the triazine without undue toxic effects. Treatment of 7-day old transplants of the Flexner Jobling carcinoma with this dose rate for 7 days produced regressions in 90 per cent of animals in 1 to 2 weeks. There was no reappearance of tumours during the next 3 months, which was taken to indicate complete cure. A similar course of treatment applied to 7 day old tumours of the Sarcoma R 39 resulted in their complete destruction in 1 to 2 weeks after the first injection.

The American strain of Wistar rat used in the present work showed little evidence of toxic effects at a daily dose level of 0·2 mg./kg. of triethylene melamine, apart from loss of appetite and weight. This latter may in turn hinder the growth of the resistant Walker tumour and the other tumours mentioned. Not only different kinds of tumour but various strains of rat vary in their susceptibility to triethylene melamine ; the growth of an occasional tumour in animals bearing a neoplasm susceptible to the drug, may be connected with some unsuspected ability of the host animal to dispose of the administered compound. It appears reasonable to suppose that ethyleneimino-compounds as a whole owe their anti-tumour activity to these highly reactive groups, so that it is difficult to conceive a protective mechanism possessed by some cells but not by the great majority in a tumour. It is equally difficult to imagine a high degree of susceptibility in one variety of neoplasm and indifference in another kind to chemical inhibitors like triethyleneimino-phosphoramide (III), which is highly soluble in water and likely to diffuse with ease into cells. Then too, there is the fact which has been established on many occasions, that the susceptibility of sensitive tumours to these drugs diminishes markedly with the age (*i.e.*, size) of the tumour. It is hoped that a study of the mechanism by which the resistant Walker tumour is derived from the original variety and an investigation of the reason for the insusceptibility of the former tumour may throw light on these interesting and important phenomena.

Only preliminary cytological studies have as yet been carried out. Inhibited nodules from the Walker carcinosarcoma treated with triethylene melamine, 24 hr. after the conclusion of a course of treatment show intact tumour cells. No mitotic figures were seen. Estimation of the mitotic index of the original and derived forms, 48 hr. after the administration of the last of 2 doses of the same drug reveals that the growth rates of the two tumours are similarly depressed. The drug would therefore appear to be exerting a cytotoxic effect on the resistant form, although the overall growth of this tumour is not much hindered by such treatment. A comparative study of the cytological effects of ethyleneimines on these tumours is continuing.

SUMMARY.

1. Administration of triethylene melamine to animals bearing 24 hr. implants of the Walker carcinosarcoma, causes complete inhibition of tumour growth. Actively growing tumours (4-5 days old) with the same treatment first regress and then remain inhibited.

2. Inhibited tumour nodules subsequently grow after a latent period of 2-3 weeks in 70–90 per cent of animals. This happens irrespective of the duration of treatment with the ethyleneimine, even though this may be continued throughout.

3. One of these tumours, selected at random, was resistant to further treatments; there was no build-up of resistance with successive courses of the drug.

4. This resistant tumour, maintained routinely for over 12 months, has shown no tendency to recover its sensitivity to triethylene melamine.

5. Cross-resistance of a similar order of magnitude is shown by this derived tumour to two other ethyleneimines.

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EXPERIMENTS ON THE INTERACTION OF POLYCYCLIC HYDROCARBONS WITH EPIDERMAL CONSTITUENTS

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Experiments on the Interaction of Polycyclic Hydrocarbons with Epidermal Constituents.

INTERPRETATIONS of the carcinogenic properties of the polycyclic hydrocarbons in terms of chemical interactions between the carcinogen and tissue constituents have been put forward by Crabtree (1945), who envisaged combination of the carcinogen with sulphur linkages as a primary phase in the process and by Boyland (1948 ; see Fig. 1 ; 1950) who suggested reaction with sulphydryl groups of enzymes necessary for metabolism of deoxypentose nucleic acid, or actual combination with nucleic acid (Boyland, 1952). Hendry, Homer, Rose and Walpole (1951) also believed that their polymerisation theory of carcinogenesis would involve combination with tissue protein. Recently, Miller and Miller (1952) have produced a good deal of evidence showing that certain polycyclic hydrocarbon carcinogens bind with skin proteins. Their results have been confirmed and extended by Weist and Heidelberger (1953) using 1 : 2 : 5 : 6-dibenzanthracene containing C^{14}. Valuable earlier evidence that 3 : 4-benzpyrene or its metabolites become intimately bound to skin proteins was given by the extensive researches of Weigert and Mottram who recovered four types of metabolic products from the organs of benzpyrene-injected mice, which they designated X_1, X_2, F_1, F_2, (Weigert and Mottram, 1946*a*, 1946*b* ; Doniach, Mottram, and Weigert, 1943 ; Weigert, Calcutt and Powell, 1947), the hypothetical radicals R_1 R_2 (Fig. 2) being "derived from the cells with which benzpyrene or benzpyrene X_1 come into contact."

The hypothesis that the production of skin cancer by such chemical agents is related to their ability to combine with tissue components at present relies almost entirely upon mouse experiments using potent carcinogens 3 : 4-benzpyrene and 1 : 2 : 5 : 6-dibenzanthracene. It seemed important, therefore, to assess the binding capacities of some polycyclic hydrocarbons allied to 1 : 2 : 5 : 6-dibenzanthracene but which are non-carcinogenic to mice, and also to determine the level of "bound" hydrocarbon, after comparable treatment, in the epidermal tissues of animals such as rats and rabbits which are less sensitive than mice to tumour induction by benzpyrene (Berenblum, 1945, 1946 ; Fieser, 1938).

EXPERIMENTAL METHODS.

Compounds used.

The fluorescence of solutions of many of the inactive or less potent skin carcinogens is much weaker than that of benzpyrene. This limits the choice of com-

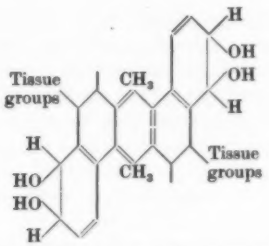

FIG. 1.—Suggested complex from 1 : 2 : 5 : 6-dibenzanthracene (after Boyland).

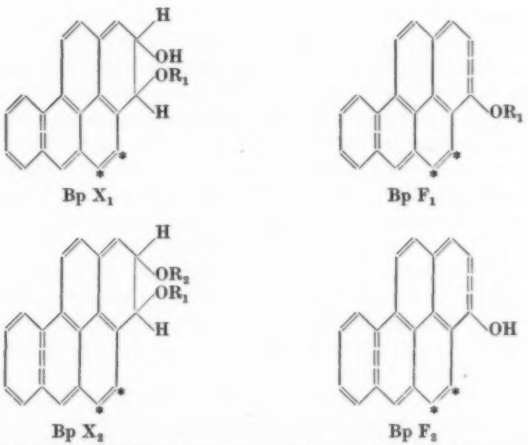

FIG. 2.—Metabolic derivatives of benzpyrene (after Weigert and Mottram, 1946*a*).

The radicals R_1, R_2 are derived from the cells with which Bp or Bp X^1, come into contact. Presumably tissue groups could also combine at the K region marked *.

pounds which are satisfactory for carrying out quantitative measurements on the very small amounts likely to be encountered. However, the fluorescence values of 2′6-dimethyl benzanthracene, 1 : 2 : 5 : 6-dibenzacridine, which are relatively weak carcinogens when applied to mouse skin, and especially of perylene which is inactive (Cook, 1932), were found to be of such intensity that 0·05 μg./ml. could be estimated with sufficient accuracy on the fluorimetric system available. The first two substances were pure specimens given by Professor J. W. Cook. The perylene was provided by Dr. A. S. Harris, Coal Tar Research Association, who had purified it by chromatography and checked it by ultra-violet spectroscopy for impurities. These were below the level of detection.

Fluorescene measurements.

The fluorescence values for solutions of the various hydrocarbons were determined by constructing curves using a Hilger " Spekker " photoelectric fluorimeter with cells of capacity approximately 11 ml., and with Filter Chance 0 × 7

inserted between the ultra-violet lamp and the solution. A combination of Filters H503, H508, H556 was introduced to reduce the light intensity on the left-hand side. A moving-mirror galvanometer 2 metres from the scale was used for the photocurrent measurements and the method of "null deflection" employed throughout. The photoelectric system was brought to a balance with the light spot at the zero on the scale in the presence of a standard fluorescent solution (usually 0·1 μg./ml.) and then a second standard solution, e.g., 0·2 μg./ml. was introduced, which resulted in reproducible deflection. The fluorescence was recorded in terms of the drum reading when the galvanometer "spot" was restored to the zero point on the scale. Between 0·1 and 0·4 μg./ml. the curves were linear for all the compounds and this range was suitable for all the experiments described.

Treatment of animal tissues.

The hair was carefully removed with clippers from an area approximately 15 sq. cm. on the back of the mice, 50 sq. cm. from rats and guinea pigs, and about 100 sq. cm. from the rabbits. The hydrocarbons were applied as 0·4 per cent acetone solution on 6 successive days using 0·2 ml. for each mouse painting, 0·5 ml. for rats and guinea pigs and about 1·0 ml. for rabbits. This solvent was chosen since it is essentially inert to the skin. The skin from the treated area was removed on the 8th day and treated as described by Miller (1951) to prepare the epidermal proteins. Pooled batches of skin from 5 animals were used for each mouse test, but a single rat or rabbit gave a sufficient amount of tissue. After separation, the epidermal layer was homogenised in a Waring blender and the protein precipitated with 85 per cent ethanol containing 10 per cent w/v trichloracetic acid, centrifuged and "washed" several times with 85 per cent ethanol which removed a good deal of "adherent" hydrocarbon.

The "moist" protein was wrapped in filter paper, extracted on a Soxhlet reflux for 24 hours with ethanol and the solvent, which then fluoresced strongly in ultra-violet light, discarded. The extraction was then continued until the alcohol extract, after evaporating to about 10 ml., showed negligible fluorescence when examined in ultra-violet light. The protein was further extracted with boiling ethanol in a reflux apparatus and finally several times with boiling benzene. Minute traces of fluorescent material could be detected in ultraviolet light and the extraction was not considered to be complete until both alcohol and benzene extracts were free from fluorescence. All organic solvents used in the experiments were distilled from clean, all-glass apparatus and checked for absence of fluorescence.

Extraction of bound hydrocarbon.

The extracted protein was dried *in vacuo* and a 25 mg. sample reflexed for 1 hour with a mixture of 2 ml. ethanol, 5 ml. 4N KOH, 5 ml. toluene, and approximately 1·5 g. zinc dust. When cool, the solution was shaken with 10 ml. benzene and the organic solvent removed after separating in the centrifuge. The aqueous portion was decanted from the zinc and again extracted several times with 10 ml. benzene. The benzene extracts which became successively less fluorescent, were combined and evaporated to 20 ml. at reduced pressure.

When this fluorescent component had been completely removed the alkaline hydrolysate was acidified to pH 4 with 7N HCl and the solution again extracted

3 times with 10 ml. benzene. It was found that, as described by Miller (1951), an extract was obtained which had the general type of fluorescence of the hydrocarbon originally applied.

The fluorescence of each solution was measured by comparing it with a standard solution of the hydrocarbon originally applied to the animal—usually 0·1 μg./ml. in benzene.

RESULTS.

The substances recovered from the hydrolysed skin proteins undoubtedly consist in part of metabolites of the compound applied and possibly of products formed during the extraction. These usually have a lower intensity of fluorescence than the present hydrocarbons, so that the values obtained from the determinations, expressed in terms of the applied substance, will be minimal. It should also be noted that the measured fluorescene was produced by a fairly broad wave band of ultra-violet light, 2500–4000 mμ.

The results obtained in a series of experiments are given in Table I. All the tests have been repeated on several animals or batches of animals and, in every case, the hydrolysis was carried out on at least two samples of dry protein. Small variations were found in the quantities of bound hydrocarbon, from different animals, or batches of animals, of the same species, but these do not affect the general conclusions which may be drawn from the tests.

TABLE I.—*Bound Hydrocarbon from Skin Protein.*

Animal.	Hydrocarbon.	μg. /25 mg. protein	
		Alkaline hydrolysate.	Acidified hydrolysate.
Mice	3 : 4-benzpyrene	(1) 1·0, (2) 1·3, (3) 0·96	(1) 0·65, (2) 0·8, (3) 0·84
,,	2′6-dimethyl-benzanthracene	(1) 1·08, (2) 1·06	(1) —, (2) 0·9
,,	1 : 2 : 5 : 6-dibenzacridine	(1) 0·94, (2) 1·0	(1) 0·62, (2) 0·6
,,	Perylene	(1) 1·0, (2) 1·2	(1) 0·62, (2) 1·05
Rat	3 : 4-benzpyrene	(1) 0·92, (2) 0·7	(1) 0·64, (2) 0·47
Rabbit	3 : 4-benzpyrene	0·75	0·74
Guinea-pig	3 : 4-benzpyrene	(1) 0·75, (2) 1·20	(1) 0·50, (2) 1·15

The relative amounts extracted from both the alkaline and acidified solutions from 25 mg. mouse protein after 6 days' benzpyrene treatment appear to be of the same order as that recovered by Miller (1951) although it is not possible to assess gravimetrically the data given by this worker since her figures are relative and apply to the instrument and filters employed. Berenblum and Schoental (1942) found that 24 hours after one intraperitoneal injection of 10 mg. benzpyrene in sesame oil into a mouse, the total blood contained about 0·8 μg. of benzpyrene. Weigert and Mottram (1946*b*) agreed with this figure and also computed that after one skin application of benzpyrene the maximum fixed hydrocarbon was present after about 8 hours and was not more than 1/100 of the amount painted on the skin.

The benzpyrene derivative extracted from acid solution is of particular interest. It possibly corresponds to the type of derivative designated as X_2 by Weigert and Mottram (1946*b*). It is not an " artefact " produced by interaction of benzpyrene with tissue or reagents during the hydrolysis since when 0·5 μg.

benzpyrene was added to denatured, fat-free, normal skin-protein and refluxed with alkali, toluene and zinc, it could be completely and quantitatively removed from the alkaline solution by benzene extraction. With the exception of benzpyrene itself, no investigations have been reported up to the present on the metabolism or metabolic products of the substances used in these experiments.

DISCUSSION.

These experiments confirm the evidence of previous workers concerning the presence of polycyclic hydrocarbon firmly bound to skin protein after applications of benzpyrene to mice, but they also show that the phenomenon is not confined to highly active carcinogens and appears to be independent of the species of animal or the presence of carcinogenic activity in the hydrocarbon.

The significance of the protein binding in the induction of cancer might, therefore, be questioned.

It was shown previously (Miller, 1951) that the amount of bound benzpyrene increased steadily in the mouse epithelium when applications were made for 6 days and then remained fairly constant. In the present tests the analyses were made at the period when benzpyrene gives a high level of bound hydrocarbon. This value is determined by the rates of formation and removal which probably vary with different species of animal and with each hydrocarbon. It is possible that hydrocarbons may combine with more than one enzyme or reactive cell constituent; only some of these combinations may be involved in the mechanism of carcinogenic transformation. Miller (1951) has put forward a number of facts which were suggested as supporting the belief that protein binding was concerned in carcinogenesis. These are (1) "The bound hydrocarbon is restricted to the epidermal layer." (2) "It appears after one application, and some proportion is still *in situ* 14 days after this." (3) "It is decreased by irradiation of the animals with ultraviolet light during the exposure period." Such features could well be associated with mechanisms not concerned with cancer induction. Moreover, there is no clear proof that the bound carcinogen is elaborated by the living cells, or is preferentially localised in the cells rather than in the intercellular tissue components. It is true that Weigert and Mottram (1946*b*) found a derivative of benzpyrene in the "cells of the Malpighian layer," and after alkaline hydrolysis recovered substances with X_2 type of fluorescence. Also Weist and Heidelberger (1953) painted mice with 1 : 2 : 5 : 6-dibenzanthracene containing C^{14}, and separated various fractions from the homogenised epithelium. These included the ribonucleic acid and deoxyribonucleic acid proteins—essentially cellular in origin—and the other soluble and insoluble proteins which are to a great extent derived from non-cellular elements. They found that the radio-activity of the bound hydrocarbon (measured as counts per min./mg.) was very similar for both these fractions. Thus protein binding by these hydrocarbons would appear to occur both inside and outside cells. The extra-cellular bound hydrocarbon might consist of metabolites "excreted" from the cells or it might be derived from dead or damaged cells.

Although the fluorescence technique was not sufficiently sensitive for accurate estimations with the small amounts of protein available, the occurence of bound hydrocarbon in cellular constituents has been confirmed by extracting the DNA and RNA skin proteins from a batch of benzpyrene-treated mice. From the 12

mg. dry RNA protein obtained, approximately 0·05 μg. of "fixed" benzpyrene was extracted and a trace was found in the DNA protein sample which, however, weighed only 2 mg.

A corroborative experiment was also carried out based on the observations of Calcutt and Payne (1953) who showed that nuclei and mitochondria isolated from the livers of mice which had been given a single intraperitoneal injection of benzpyrene in finely dispersed suspension, contained fluorescent hydrocarbon 2 hours to 21 weeks after the injection. They found that after 3 or 4 extractions of the isolated nuclei with acetone containing 30 per cent water, no more fluorescent substance was removed. They believed, therefore, that benzpyrene injected in this way is rapidly transported to the mitochondria and nuclei and is held there in an unchanged state for prolonged periods. They did not examine the effect of alkaline hydrolysis on alcohol-extracted nuclei.

The experiment was repeated in this laboratory but the preliminary extractions were made as described previously for the skin tissues and continued with alkaline hydrolysis. The nuclei were isolated from the livers of 5 mice by the citric acid technique 48 hours after injecting the animals intraperitoneally with 10 mg. benzpyrene in 0·5 ml. aqueous colloidal "solution." The clean preparation of nuclei was thoroughly extracted with alcohol and when no more fluorescent material could be removed by this reagent, 15 mg. of the dry nuclei were decomposed with alkali in the usual way. A small amount of fluorescent material was extracted from the hydrolysate but nuclei from control mice did not yield comparable fluorescent extracts at any stage.

This experiment showed that at least a portion of the benzpyrene can be fixed *in vivo* by nuclear components. It was repeated, therefore, employing perylene, and, after alkaline hydrolysis, an extract with fluorescence characteristic of perylene was obtained from the separated, alcohol-treated nuclei. Both these hydrocarbons, therefore, combine with cell constituents so that the property does not appear to be a special function of compounds with carcinogenic activity.

SUMMARY.

The epidermal proteins from mice, rats, rabbits and guinea pigs have been prepared after treating the skin with 6 daily paintings of 0·4 per cent 3 : 4-benzpyrene in acetone. The free hydrocarbon was thoroughly removed and the "bound hydrocarbon" was extracted after hydrolysing the protein with KOH and measured fluorimetrically.

Although these species of animals vary considerably in their response to this hydrocarbon as a carcinogenic agent the amounts of bound hydrocarbon obtained from 25 mg. samples of extracted and dried tissue were very similar in all instances.

Other polycyclic hydrocarbons which are less carcinogenic to mice, e.g., 2'6-dimethyl-benzanthracene, and perylene which is non-carcinogenic, have also been tested, and in all instances comparable amounts of bound hydrocarbon could be extracted from the alkaline hydrolysate.

Using similar extraction techniques a small amount of fluorescent, bound hydrocarbon could be removed from the nuclei isolated from livers of mice 24 hours after a single intraperitoneal injection of either 3 : 4-benzpyrene or perylene.

It is concluded that further evidence is needed to substantiate the view that

hydrocarbon-protein binding in the cells is the essential factor in the chemical induction of skin cancer.

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NEW DATA ON THE ULTRACHONDRIOMA

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In 1950 Oberling, Bernhard, Braunsteiner and Febvre noticed the presence of ultramicroscopic granules and rods in leukoblasts of human leukemias and discussed their possible relationship with corpuscular elements earlier observed by Porter and Thomson (1947 ; 1948) in cells of rat cysticercus sarcoma.

Further studies (Oberling, Bernhard, Guerin and Harel, 1950; Oberling, Bernhard, Febvre and Harel, 1951, 1952*a*, 1952*b*) revealed a great polymorphism of these elements, their evident similarity with mitochondrial structures and their frequent occurrence not only in neoplastic but also in normal cells. These results led Oberling and his co-workers to consider them as a submicroscopic chondrioma or ultrachondrioma.

Since then, Selby and Berger (1952) have described such formations in tissue cultures of human carcinomas while Porter and Kallman (1952) found them also in rat fibroblasts derived either from embryos or from sarcomas and called them "growth granules."

We have further studied these particles in the hope of elucidating their nature, and more especially their possible relationships with virus infections and with cancer.

MATERIAL AND METHODS.

The methods used have already been described (Bernhard, Febvre and Harel, 1950 ; Harel and Bussmann, 1952). They take advantage of the spontaneous adhesion on plastic membranes of the free cells present in physiological fluids such as blood, cerebro-spinal fluid, peritoneal and pleural effusions.

In brief : glass slides coated with formvar are placed in contact with the fluid (with addition of heparin if necessary) in an incubator at 37° C. for 15 to 60 minutes.

The slides are then rinsed with Tyrode solution at 37°, fixed 10 minutes to several hours in acid osmic vapour and washed thoroughly in distilled water. The formvar is stripped from the slide, mounted on grids and allowed to dry at room temperature for at least 24 hours.

Photographs were taken with a Trüb-Taüber electron microscope at 60 kV. We adopted stringent criteria for purposes of comparison and considered only undamaged cells, that is to say cells having clear and well defined chondriosomes and with a satisfactory cytoplasmic transparency. As the appearance of a cell may vary from one area to another (because of differences in spreading, lipid constituents, etc.) we only considered the best portions. Counts were made in each cell of osmiophilic bodies less than 150 mμ. in greatest diameter, in an area of 100 μ^2. At least a few such bodies are to be found in every cell, but to consider them as true ultrachondrioma we adopted as a minimum 10 such particles

showing a certain degree of polymorphism (to distinguish them from merely lipidic granules) per 100 μ^2. For each preparation at least 1000 cells were counted. We also made optical controls with the usual staining methods.

OBSERVATIONS.

Human leukocytes.

We observed two series of human normal blood preparations and used the same method of statistical analysis for 2 new cases of typical acute myelocytic leukemias (55,000 and 112,000 cells per mm.^{3} respectively).

Results are summarized in Table I.

TABLE I.—*Human Leukocytes.*

	Number of preparations.	Percentage of satisfactory cells.*	Percentage of polymorphonuclear leukocytes.	Percentage of lymphocytes.	Percentage of monocytes.	Percentage of immature cells.	Percentage of cells containing obvious ultrachondrioma.
Normal blood	4	30	90 to 95	4 to 6	1	0	5 to 15
	2	50	Ditto	Ditto	Ditto	0	50
Leukemias	2	70	10 to 25	5 to 10	10	55 to 75	35

* Well preserved and well spread cells as defined previously for electron microscopy.

Fig. 1 shows a typical aspect of the ultrachondrioma in a polymorphonuclear leukocyte of normal blood. The most common forms are granules (grouped in chains, pairs, or clusters) and rods. Rather often one may observe club and dumbbell like, or comma-shaped forms, and very rarely long filaments.

Ultrachondrioma is found in immature as well as in adult leukocytes and is not therefore an attribute of immature cells.

Whether differences of a purely quantitative nature exist between normal and pathological leukocytes is a possibility which remains to be explored.

Spontaneous effusions.

We observed 39 new cases of effusions in man (ascites, pleurisies and pathological cerebro-spinal fluids).

Results are summarized in Table II.

Fig. 3, 4, 9 show typical aspects of the ultrachondrioma in mesothelial cells of effusions in man and malignant reticulosis of the mouse of Guerin (1949), which frequently occurs in our strain (Table III). The same submicroscopic corpuscles as in leukocytes are encountered, but frequently they appear as bent or twisted filaments which may have swellings or a beaded structure. Sometimes these filaments are extremely long. Their thickness may vary from 30 mμ. up to 150-200 mμ. which is the size of the finest chondrioconts. Sometimes they occur as tangled masses interspersed with spherules. They may aggregate in groups of two, three or more, or may appear to arise from an ordinary chondriocont.

The statistical analysis shows that those submicroscopic bodies are not a characteristic only of malignant effusions but are seen in various inflammatory or circulatory disturbances. The quantitative differences which might be inferred

TABLE II—*Human Effusions*

Cases.	Total number of cases.	Number of cases showing satisfactory cells.*	Percentage of malignant cells.†	Percentage of cells containing obvious ultra-chondrioma.
Cancerous fluids :				
Carcinoma of the breast.	13 pleurisies 2 ascites	3	3 to 15	5 to 25
Carcinoma of the ovary	1 pleurisy 3 ascites	2	6 to 12	10 to 15
Hodgkin's disease	3 pleurisies	1	0	0
Myosarcoma	1 pleurisy	1	10	90
Endothelioma	1 pleurisy	1	90	15
Glioblastoma	1 cerebro-spinal fluid	0	50	0
Carcinoma of the cervix.	1 ascites	0	10	0
Non-cancerous fluids :				
Cirrhosis	8 ascites 1 pleurisy	2	0	5 to 30
Tuberculosis	1 ascites	0	0	0
Meningoencephalitis	2 cerebro-spinal fluid	1	0	20

* Well preserved and well spread as defined previously.
† As determined by optical methods.

TABLE III.—*Effusions in the Mouse.*

	Total number of cases.	Number of cases showing satisfactory cells.	Percentage of malignant cells.	Percentage of cells containing obvious ultra-chondrioma.
Ascites in malignant reticulosis	9	8	12 to 25	5 to 60
Control ascites induced by Kiesel-guhr	5	2	0	5 to 30

from Table II are in our opinion not relevant on account of the great discrepancies between effusions especially so far as the percentage of unaltered cells is concerned.

Morever it appeared to us quite impossible to make sure whether in the neoplastic effusions the granulo-filamentous bodies were predominantly located in malignant cells. The distinction of cancer cells is impossible under the electron microscope with the applied methods.

Induced effusions in animals.

Because of these difficulties we made a comparative study of two types of induced effusions in animals of similar strain, age and diet.

I.—Cancerous effusions in the rat were derived from a fibroblastic sarcoma isolated and transplanted serially as an ascitic tumor according to the methods used by many workers (e.g., Klein, 1951). On the fifth intra-abdominal transplantation an abundant ascites developed after 15 to 20 days. Cytological examination showed a marked predominence of maligant cells, and subcutaneous injections of the fluid into other rats induced solid tumors.

II.—Two control series of induced effusions.

Control I : irritation of the peritoneal serosa by three to five daily injections of 1 per cent Kiesleguhr suspensions in Ringer solutions into rats and mice.

Control 2 : injections of colloidal radioactive gold into rats, (Harel, 1953) according to the method used by Hahn, Jackson and Goldie (1951) for dogs. After some months distinctive hepatic lesions appeared with effusions rich in mesothelial cells but containing few leukocytes. In order to avoid secondary infection the fluid was withdrawn as soon as the effusion was sufficiently developed.

Results are summarized in Tables III and IV.

TABLE IV.—*Induced Effusions in the Rat.*

	Total number of cases.	Number of cases showing satisfactory cells.	Percentage of malignant cells.	Percentage of cells showing obvious ultrachondrioma.
Cancerous effusions :				
Ascites tumor Sarcoma T. 395	7	5	30 to 60	60 to 100
Non-cancerous effusions :				
Effusions induced by Au 198	8	8	0	60 to 100
Effusions induced by Kieselguhr	5	2	0	10 to 30

The statistical study in these series appears highly conclusive because a comparison could be made between effusions containing a high percentage of neoplastic cells and others containing none.

In some well chosen preparations the amount of well preserved cells malignant or non-malignant, containing obvious ultrachondrioma was nearly 100 per cent. It was 100 per cent in some preparations of cancerous ascites as well as in preparations of effusions induced by Au 198. In effusions induced by Kieselguhr the inflammatory reaction was often predominent and inflammatory cells spread rather badly on the formvar.

EXPLANATION OF PLATES.

FIG. 1.—Leukocyte of human normal blood. Exceptional abundance of ultrachondriomal structures. About × 5,500.

FIG. 2.—Vegetal chondrioma. Epidermis of allium Cepa. Fixed with Meves. Coloured with Regaud's hematoxylin (optical microscope).

FIG. 3.—Cell of peritoneal fluid of the mouse. Malignant reticulosis of Guerin (electron microscope). Compare with vegetal chondrioma of Fig. 2 (similar structures are observed but they are five times smaller). × 8,400.

FIG. 4.—Vegetal chondrioma. Allium Cepa epidermis stained *in vivo* with Janus green. (Optical microscope.)

FIG. 5.—Cell from human neoplastic pleurisy (electron microscope). Ultrachondrioma appears as long fine filaments (compare with vegetal chondrioma of Fig. 4). × 5,800.

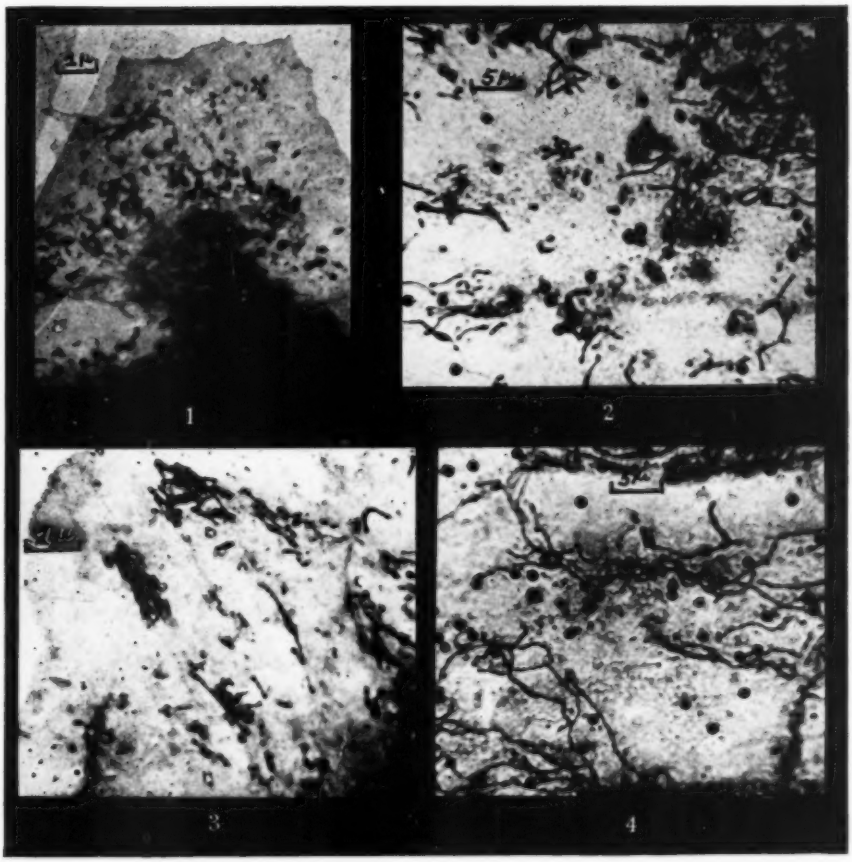
FIG. 6.—Cell of peritoneal fluid of the mouse. Malignant reticulosis of Guerin. The largest filaments do not exceed 200 μu. in width, the finest ones 50 μu. × 4,500.

FIG. 7.—Mesothelial cell. Peritoneal fluid of the rat. (Effusion induced by Kieselguhr.) C, ordinary chondriosomes. u, ultrachondrioma. × 9,000.

FIG. 8.—Cell from ascites of the rat (cirrhosis induced by Au 198). M, ordinary mitochondrioma. u, ultrachondrioma. e.r., endoplasmic reticulum. f, filaments whose osmophilia is intermediate between those of u and e.r. × 14,000.

FIG. 9.—Cell from human pleurisy. All kinds of transitions both in size and structure are seen between ordinary chondriosomes and the finest bodies. × 8,000.

FIG. 10.—Cell of Murray-Begg endothelioma in tissue culture (perinuclear zone). C, ordinary chondriosomes. u, ultrachondrioma. × 8,000.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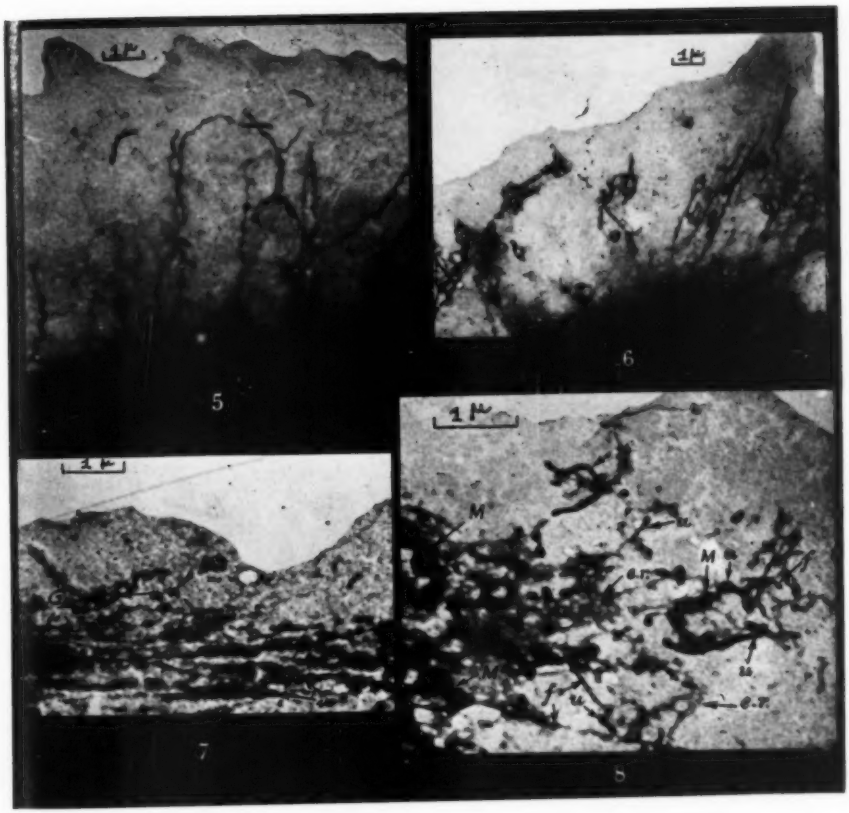
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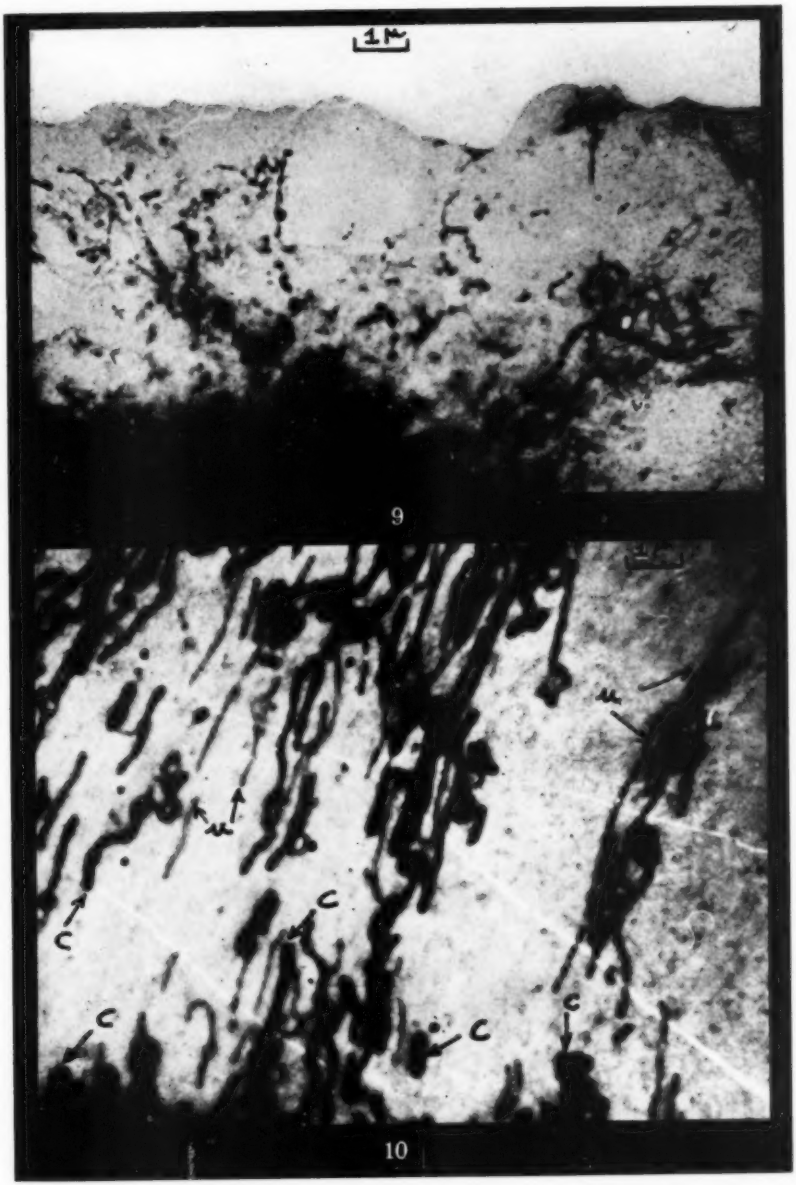

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Harel and Oberling.

Certain figures were identical with those obtained by Porter and Kallman (1952) using different methods and material, but on the whole the formations we observed were more polymorphic than those described by these authors.

Infection with rabbit myxoma virus.

In order to determine the possible action of virus on the submicroscopic structures of effusion cells we induced pleural effusions in 6 rabbits by 5 daily injections of Kieselguhr. In 3 rabbits (carefully isolated) this effusion was infected with myxoma virus (intrapleural injection of 2 c.c. of 5 per cent solution of filtered ground myxomatous tissue in Ringer solution). The pleural fluid was withdrawn 24 hours, 3 days and 7 days after the infection both in infected and control animals. Infected animals died after 8–10 days with typical symptoms of myxoma and an abundant pleurisy.

The infectivity of effusion cells was demonstrated by washing them in saline several times after centrifugation, resuspending the pellet and injecting it into 3 further animals who died after the same time interval with obvious myxoma.

As far as quantitative and qualitative behaviour of ultrachondrioma is concerned no significant difference was found between myxoma infected cells and non-infected cells in Kieselguhr effusions.

Avian tissue cultures.

In addition to this study of effusions we have examined tissue cultures of the avian Murray-Begg endothelioma, using hanging-drop cultures prepared for electron microscopy by the usual methods. Recently we have observed typical ultrachondrioma exclusively in the form of chondrioconts in a few cells in which the ordinary chondrioconts were particulary abundant and well preserved.

DISCUSSION.

Our ideas concerning the mitochondrial nature of the described submicroscopic structures are mainly based on the following facts :

They have the same osmophilia as chondriosomes and transitional structures are frequently observed. We fully realize that transitional figures as such are of no meaning when they are occasionally found, because they may be observed between all kinds of structures. But when those figures are an almost constant finding, when it becomes impossible to establish a distinction between chondrioconts and those structures on any other basis than the rather arbitrary one of size and thickness, then it seems difficult to deny the mitochondrial nature of the described structure.

They have the same characteristics and the same polymorphism as certain types of mitochondria (especially in plant cells). The filamentous forms, the structures suggesting divisions, the great variations from one cell to another are absolutely identical, the only difference being the size of the elements (compare Fig. 2 and 3 and Fig. 4 and 5).

There may be some unwillingness by cytologists to admit the mitochondrial nature of formations unless they stain with Janus green and exhibit functional capacities generally ascribed to those structures.

For obvious reasons these two postulates cannot be met for the time being, but it may be remembered that mitochondria were recognised as a cytological entity long before Janus green had shown its utility as a mitochondrial stain and

before anything was known about functional abilities of the chondrioma. Even the specifity of Janus green stain depends on certain criteria (Showacre, 1953).

Furthermore there is a question of principle involved in the discussion about the significance of these structures. If we require for the identification of ultramicroscopic structures the same characteristics as those used in classical cytology, if certain mitochondria can no longer be considered as such because their size prevents the use of Janus green and the application of more or less debatable functional tests, then we shall have two cytologies : one optical and one electronical, which is not satisfactory. It is highly improbable that cytoplasmic constituents are of a different nature simply because they are too small to be seen with the ordinary microscope.

On the contrary it should be our aim to show the links between microscopic and ultramicroscopic structures. This has been done with great benefit with basophilic structures, ergastoplasm and intracytoplasmic network. It would be curious if the same did not hold true for the mitochondrial structures.

Furthermore the concept of mitochondria which are invisible with the optical microscope is not new and it can account for certain results.

Many cytologists consider the chondrioma as fixed both in amount and morphology. But this stability is dependant upon the material studied, and it is in fact true of specialized tissues observed under standard conditions. Botanists have long noted the morphological variations of the chondrioma in active cells (Guilliermond, 1934 ; Gautheret, 1950). Noël (1924) and many others have made similar observations with animal cells.

The researches of Levi (1934), Lewis and Lewis (1915), etc., seemed in favour of the *de novo* appearance of mitochondrioma in cells of tissue cultures. Recently Chevremont and Frederic (1952) observing tissue culture cells during mitosis with the phase contrast-microscope, noted the transformation of chondrioconts into very minute rods and granules which resemble the structures described here. These formations sometimes disappeared and appeared again in daughter-cells and then growing thicker and longer, reconstituted ordinary chondriosomes.

The existence of the ultrachondrioma may well explain the apparent appearance *de novo* of mitochondrioma as a result of their regeneration from the ultrachondrioma.

It is not impossible that the absence of the ultrachondrioma in many cells may be more apparent than real. The degree to which ordinary mitochondria are susceptible to variations of physicochemical conditions such as pH, osmotic pressure, is well known. Minor variations of conditions that are uncontrollable by present methods might cause a cavitation of the ultrachondrioma that would make it impossible to distinguish from the surrounding endoplasm. Actually we never observed ultrachondrioma in cells where swelling of the ordinary chondrioma occurred. Furthermore we sometimes found ultrachondrioma in the perinuclear region when very favourable conditions permit observation in this area. But usually the thickness and high lipid content of this area in spread cells prevent its study with the electron microscope. The use of improved tissue sections may later allow us to solve this problem of the universality of the ultrachondrioma.

Relations of the ultrachondrioma with the other cytoplasmic constituents.

The ultra-mitochondrial structures are usually quite distinct from the endoplasmic reticulum of Porter which is in some way connected with the microsomes

of Claude (1946, 1949) or the " granules ribonucleo-proteiques " of Brachet (1948). This endoplasmic network presents actually many and varied aspects in spread cells ; sometimes as a more or less dense mass from which moniliform filaments often arise. In certain cells especially leukocytes, this reticulum cannot be observed, even after prolonged fixation (24 hours) with osmic acid.

In a very few cases photographs seem to indicate a series of transitional states between the ultrachondrioma and the endoplasmic reticulum. Some filaments appear to have an osmoplilia intermediate between the two, and denser forms may sometimes be distinguished in the middle of endoplasmic masses. Can we consider these apparent transitions as a morphological confirmation of the biochemical researches which postulate the existence of a series of intermediates between the " microsomal " fraction and the mitochondrial fraction (Chantrenne, 1947 ; Jeener, 1948 ; Jeener and Szafarz, 1950 ; Novikoff, Podber, Ryan and Noll, 1953)? We consider however that in our material the intermediary figures between endoplasmic reticulum and ultrachondrioma are too scarce to permit any general conclusion concerning the possible relationship between the two structures.

Role of the ultrachondrioma.

Contrary to the conclusions of Selby and Berger (1952) we have never observed any predominance of any special type of the ultrachondrioma in cancer cells, and as we have shown, all our results indicate that those structures are not directly related to the process of cancerisation. Moreover so far as induced effusions in the rat are concerned there is no significant quantitative difference between malignant and normal cells.

The previous suggestion of a role of the ultrachondrioma in the proliferation of virus appears rather unlikely since the experiments made to verify it proved negative. These negative findings should not however be considered as absolute proof that the ultrachondrioma may not have some as yet unknown connection with the multiplication of viruses, for the existence of latent viruses is well known.

Porter and Thompson (1947, 1948) consider that the submicroscopic formations which in fact they observed almost exclusively in actively proliferating cells are " growth granules." However we have found an abundance of similar corpuscles in ordinary mesenchyme cells from adult animals and in leukocytes.

Their formation or development seem to be linked to certain stages of the cell life rather than to the growth capacity of the original tissue. The great variation of the ultrachondrioma from one cell to another and the occasional predominance of one type or another (e.g., long filamentous forms on the one hand, short granulo-filamentous forms on the other) within cells in the same preparation while yet other cells show intermediate forms may lead to suppose the existence of some sort of cycle between the chondrioma and the ultrachondrioma.

The predominance of one stage or another may result from difference in the metabolism or development of the cells.

CONCLUSION.

Ultrachondrioma appears as a well characterized cytoplasmic component which is observed in normal as well as in malignant cells. Its occurrence in cells such as leukocytes of adult type or macrophages indicates that it is not specifically related to growth phenomena. Its significance is yet unknown.

There are two fields in which the existence of the ultrachondrioma raises special problems.

In cytology, the presence of corpuscles resembling viruses in normal (or presumably normal) cells should be a warning against mistaken interpretations and will complicate the task of those workers who are engaged on the problem of the interaction of cell and virus.

In biochemistry it introduces a new factor into the study of cytoplasmic fractions isolated by ultracentrifugation. It seems obvious that a large part of the ultrachondrioma will be deposited in the so-called microsomal fraction and would therefore lead the erroneous conclusions concerning the homogeneity of this fraction.

The concept of the ultrachondrioma limited though it appears should entail a critical revision of some of the present data of cytology.

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SOME REACTIONS OF SERA FROM NORMAL AND TUMOUR-BEARING ALBINO RATS WITH 2, 3, 5-TRIPHENYLTETRAZOLIUM CHLORIDE.

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ACCORDING to Jensen, Camarata and Huggins quoted by Huggins (1949), sera from cancer patients are generally less effective than normal sera for reducing 2, 3, 5-triphenyltetrazolium chloride (TTC) to the red-coloured triphenylformazan. A polarographic investigation has now been made of the TTC reaction with sera from normal and tumour-bearing albino rats of the Wistar strain and the diminished capacity of cancer serum for reducing TTC has been confirmed.

In addition, it has been found that under certain experimental conditions with TTC, tumour and normal sera give distinctly different colour reactions.

EXPERIMENTAL.

(1) *Correlation between TTC-reducing Capacity of Rat Serum and Tumour Weight.*

Animals.—Serum was obtained from normal albino rats and from rats bearing 12 day old transplants of Walker tumour (W) or dibenzanthracene sarcoma (Rb/3) and stored at 0° C. overnight before use, unless otherwise stated. The diet of the animals (Sheffield University Field Laboratories stock) and the method for obtaining serum have already been described (Neish, 1953). Tumours were dissected out and weighed to the nearest 0·5 g.

Reagent.—2, 3, 5-Triphenyltetrazolium chloride was prepared according to Mattson, Jensen and Dutcher (1948) and a stock solution of the reagent (0·1 per cent w/v in distilled water) was stored in the dark.

Method.

Serum (0·1 ml.) was mixed with 0·1 per cent TTC reagent (2 ml.) in a pyrex test tube (1 cm. diameter), N/10 KOH (1 ml.) was added and the mixture heated on a water bath at 80 ± 0·5° C. for 10 minutes. The reaction mixtures (which include a blank containing 0·1 ml. of water instead of serum) were chilled in cold water and then poured into tubes containing glacial acetic acid (1 ml.). The mixtures which had a pH of about 2·5 were polarographed as follows :

The test solution, into which dipped a dropping mercury cathode, was connected to a saturated calomel reference anode (S.C.E.) by salt-agar bridges (saturated KCl in 3 per cent agar-agar) and the solution deoxygenated by passing a stream of water-washed oxygen-free nitrogen through it for 10 minutes. The dropping mercury electrode had the following characteristics : at 20°C, in 0·1 N KCl (open circuit), $m = 0{\cdot}003118$ g./sec. and $t = 1{\cdot}83$ sec, with head of mercury, 50 cm. (For explanation of the polarographic terms and procedures, see Müller, 1951.)

Polarograms were recorded at 20° C. (constant temperature room) by means of a Cambridge Polarograph using galvanometer sensitivity 1/100 with zero damping.

TTC gave a well-defined reduction wave with $E_{\frac{1}{2}} \sim -200$ to -250 mv. versus S.C.E. The height of this wave was found to be directly proportional to the concentration of TTC up to maximum strength utilised in the present experiments. The percentage TTC reduced by serum was estimated with reference to the wave height of TTC in the blank reaction mixture. Triphenylformazan gave no reduction wave in the range 0 to $-1{\cdot}2$ volts under the present conditions.

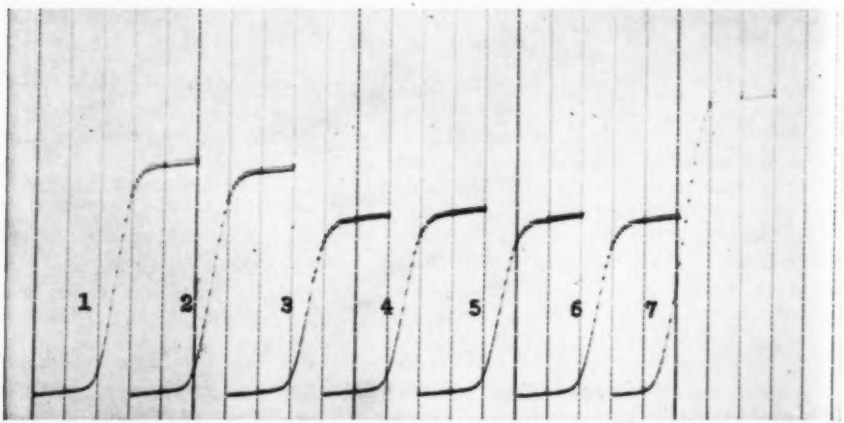

FIG. 1.—Polarograms illustrating reduction of TTC by tumour and normal sera. Each polarogram begins at 0 volts. Abscissa interval = 100 millivolts.

Polarograms 1 to 3, sera from rats bearing Rb/3 tumours of size 18·6, 21·5 and 0·6 g./100 g. body weight respectively.
Polarograms 4 to 6, sera from normal rats.
Polarogram 7, TTC Blank.

Polarographic reduction of TTC has already been described by Doskočil (1951).

Results.

Typical polarograms showing the influence of tumour and normal sera on reduction of TTC are given in Fig. 1.

Reducing properties of sera from 27 transplant animals [14 male W (2·8–20·3 per cent tumour weight), 5 female W (8·1–22·8 per cent), 6 male Rb/3 (0·6–20·5 per cent) and 2 female Rb/3 (3·4 and 18·8 per cent)] and from 23 normal animals (19 males, 4 females) were examined.

The results are plotted against tumour weight percentage

$$\left(\frac{100 \times \text{tumour weight (g.)}}{\text{total body weight (g.)}}\right)$$

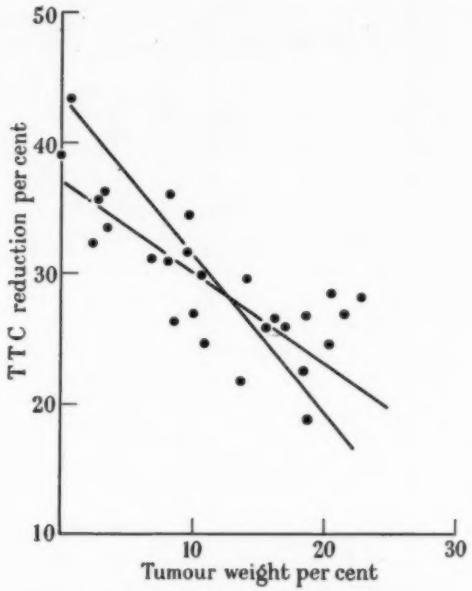

FIG. 2.—Scatter diagram showing negative linear correlation between percentage TTC reduction by serum and percentage tumour weight. Regression lines have been constructed from the regression equations:

$$x-\bar{x}=r\left(\frac{S.D._x}{S.D._y}\right)(y-\bar{y}) \text{ and}$$

$$y-\bar{y}=r\left(\frac{S.D._y}{S.D._x}\right)(x-\bar{x})$$

Here x = tumour weight per cent.
y = TTC reduction per cent.
$\bar{x}=12\cdot3222$ $S.D._x=6\cdot4315$
$\bar{y}=28\cdot5852$ $S.D._y=5\cdot8601$
$r=-0\cdot748$

Equations are:
$x=-0\cdot8206y+35\cdot7792$
$y=-0\cdot6813x+36\cdot9803$

in the scatter diagram, Fig. 2, from which it will be seen that there is a negative linear correlation between percentage TTC reduced and percentage tumour weight. The correlation coefficient (r) was calculated in the usual way (Moroney 1953) and found to be $-0\cdot75$,

$$\left[t \text{ test} = \frac{r\sqrt{N-2}}{\sqrt{1-r^2}} = 5\cdot63 \ (N = \text{number of animals})\right]$$

highly significant at the 0·1 per cent level.

Reducing capacities of tumour and normal rat sera are shown in Fig. 3. For normal animals, the mean percentage TTC reduction was 38·96 (range: 34·1 to 42·0; S.D. = 2·48).

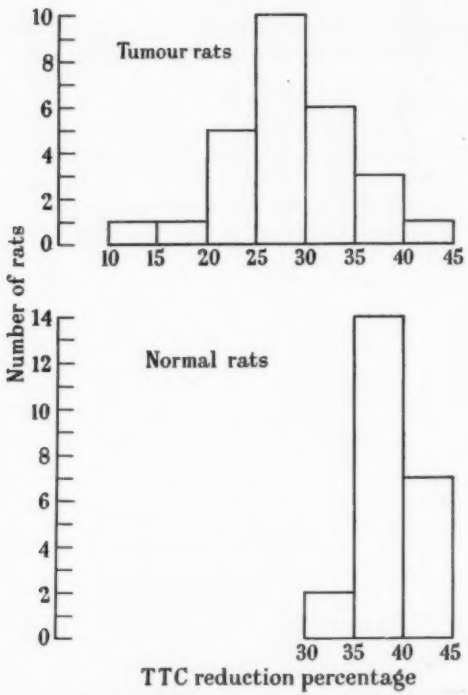

FIG. 3.—Histograms showing the capacity of tumour and normal rat sera for reducing triphenyltetrazolium chloride.

(2) *Colour Differences between Tumour and Normal Sera in a Modified TTC Reaction.*

Serum mixtures with TTC were prepared as outlined in Section (1) but this time they were heated for 90 seconds at 90–95° C., chilled in water and poured into glacial acetic acid (1 ml.). Although the mixtures just after heating had about the same intensity of red colour (triphenylformazan), the colours observed after cooling and acidification were often quite different. Thus normal serum mixtures were purple in transmitted daylight and appeared turbid pink in reflected light. On the other hand, tumour sera generally gave rise to clear red solutions. Polarographic examination of these mixtures showed that about the same proportion of TTC had been reduced in each case so that the colour differences between tumour and normal groups should be attributed perhaps mainly to colloidal effects. Results of a typical experiment are given in Table 1.

The 4-day-old Walker transplants were probably growing actively since control tumours inoculated at the same time grew to a large size in 2 weeks. It will be seen that a marked effect may be obtained even with small tumours.

Table II summarises the colour reactions that have been obtained with normal and tumour sera and gives in brackets the tumour size ranges of the animals concerned.

TABLE I.—*Colour Reactions given by Tumour and Normal Sera in presence of TTC. (Period of Heating: 90sec. at 90–95°c)*

Expt. No.	Serum donor.	Tumour size (g/100g. total body weight).	Age of transplant (days).	TTC reaction. Colour.*	Turbidity.*	Polarographic wave height (mm.).
M45†	Walker	18·3	11	Red	*Nil*	41·6
	,,	15·0	11	,,	,,	42·3
	,,	0·6	4	Pink	slight	42·2
	,,	0·5	4	,,	,,	42·5
	Normal	—	—	purple	very turbid	41·9
	,,	—	—	,,	,, ,,	43·4
M 46‡	Rb/3	5·5	13	,,	turbid	—
	,,	7·7	13	pink	,,	—
	Normal	—	—	purple	,,	—
	,,	—	—	pale purple	,,	—
	Blank (0·1 ml. water in place of serum)			colourless	—	47·2

* Colour and turbidity in transmitted and reflected daylight 5 minutes after acidification.
† Sera stored 48 hours at 0° C.
‡ Sera stored 24 hours at 0° C.

TABLE II.—*Summary of TTC Colour Reactions with Normal and Tumour Sera. (Period of Heating: 90sec. at 90–95°c.).*

Number and kind of rats.	Number of sera giving Red colour.	Purple colour.
27 Walker transplants	24 (0·5 to 25·7%)	3 (0·2 to 0·5%)
10 Rb/3 transplants	9 (4·5 to 32·3%)	1 (5·5%)
23 normals	2	21

Experiments with pure bovine plasma albumin solutions in place of serum gave very feeble or no colour reactions until dextrose was included in the mixtures. When 0·02 ml. samples of 0·1 per cent dextrose were added to TTC mixtures containing 0·1 ml. albumin solutions 1·5 to 6 per cent) the colours developed ranged from blue to purple. Thus a decrease in the albumin content of tumour serum is probably not responsible for the red colouration with TTC. Perhaps the albumin-globulin ratio of the serum influences this TTC colour reaction.

(3) *Behaviour of Serum—TTC Mixtures Exposed to Light.*

When aqueous solutions of TTC are kept in daylight they develop a red colour (compare Hauser, Jerchel and Kuhn, 1949*a*). It was decided to examine the effect of normal and tumour rat sera on this photochemical process. Samples of sera (0·1 ml.) were mixed with 0·1 per cent TTC reagent (2 ml.) in open pyrex test tubes (1 cm. diameter) and exposed to daylight. The tubes stand on a white sheet and are viewed from above. Serum hastened colour development in illuminated mixtures, the colours being marked after 1 day. Control solutions containing no serum required 2 to 3 days to develop a pink colour while serum mixtures and controls kept in the dark exhibited no colour even after 1 week.

After exposure to daylight for 2 days tumour serum mixtures were coloured pink whereas normal serum mixtures, orange-pink after 1 day, were orange-

yellow in colour. Of 10 tumour sera, 9 (tumour range, 3·4 to 22·8 per cent) showed pink colours and 1 serum (8·1 per cent) gave the orange-yellow reaction. Six normal sera each gave orange-yellow colours. In time, all colours faded to pale yellow and the solutions fluoresced blue in ultra-violet light. The initial colour changes appear to be related to the power of the sera for reducing TTC (Section (1)), the least active tumour sera giving the most intense red-pink colours.

Some polarographic studies were made of 2-day-old illuminated mixtures (2ml. samples treated with glacial acetic acid (1 ml.) followed by N/10 KOH (1 ml.)). No difference was found with respect to TTC wave height between tumour and normal serum-TTC mixtures and the illuminated control but a new wave was observed in each solution which had been exposed to light. This wave was never observed in the TTC-alkali reaction mixtures described in Sections (1) and (2), or in control mixtures kept in the dark. The new wave occurred at ~ -550 mv. versus S.C.E. and it was found to be due to another photochemical reaction product of TTC, namely the 2, 3, diphenylene-5-phenyl tetrazolium compound (blue fluorescent in ultra-violet light) described by Hausser, Jerchel and Kuhn (1949*a*) and by Kuhn and Jerchel (1952). The extent of formation of this compound appears to be uninfluenced by the presence of serum but serum suppressed the maximum which occurs on its polarographic wave in protein-free solutions. Further details of these polarographic studies will be presented elsewhere.

It may be remarked that, while the last described TTC-serum colour reactions are perhaps dependent on colloidal effects, the possibility should be kept in mind that the red-yellow colours displayed by cancer-normal sera may be connected with the known photochemical cis-trans isomerisation of red triphenylformazan (Hausser, Jerchel and Kuhn (1949*a* and 1949*b*)) to the yellow variety.

SUMMARY.

(1) Sera from rats bearing transplanted tumours are less effective than normal rat sera for reducing 2, 3, 5-triphenyltetrazolium chloride (TTC) as determined polarographically. There is a highly significant negative linear correlation between percentage TTC reduced and percentage tumour weight.

(2) Cancer and normal rat sera gave distinctly different colours in two types of reaction with TTC.

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INCORPORATION OF ^{32}P AND ADENINE ^{14}C INTO DNA BY HUMAN BONE MARROW CELLS *IN VITRO*.

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In connection with work on the isotope uptake by normal and malignant cells, the synthesis of desoxyribose nucleic acid (DNA) by normal and leukaemic bone marrow and blood cells was investigated. This paper reports the results obtained on the incorporation of ^{32}P orthophosphate and adenine-8-^{14}C into DNA by human bone marrow cells *in vitro*, and the effect of a single dose of 5000 r X-ray irradiation on DNA synthesis.

METHODS.

The technique for culturing human bone marrow *in vitro* has been described in detail elsewhere (Lajtha, 1952*a*). Cell suspensions were cultured in a liquid medium and the isotope was added after the cultures were set up. At the end of the culture period (3–26 hours) smears were made from the cultures and stained autoradiographs were prepared for differential counts and grain counting, using a high resolution stripping film technique (Lajtha, 1952*b*, 1954*a*)

In nearly all experiments described here the isotope was added so as to have a concentration of 5 μc./ml. medium ; ^{32}P orthophosphate (as supplied by A.E.R.E., Harwell, in a pH 7 phosphate buffer) was used. Lower concentrations of ^{32}P failed to give satisfactory autoradiographs. In a few experiments 10 μc./ml. medium ^{32}P was used, but the general background on the autoradiographs was then rather high due to scatter and cross fire. The concentration of adenine-8-^{14}C in all experiments was 0·5 μc./ml. medium. The specific activity of the adenine ^{14}C sulphate, as supplied from the Radiochemical Centre, Amersham, was 10 μc./mg.

All smears were alcohol fixed, and, to obtain autoradiographs from DNA only, one set of smears from each culture bottle was treated with N HCl at 60° C. for 6½ minutes. This degree of hydrolysis was found to remove all non-DNA phosphorus as well as all non-DNA adenine from the cells, while leaving DNA phosphorus and DNA adenine *in situ*, thus obviating the necessity for the use of the enzyme ribonuclease (Lajtha, 1954*b*). Adenine ^{14}C administered to cells appears both as labelled adenine and labelled guanine in the DNA (Hamilton, 1953). No attempts to separate labelled adenine from labelled guanine have been made in the experiments ; the activity of the DNA has been measured as a whole.

The autoradiographs were exposed for 10–14 days. All autoradiographs belonging to the same experiment were coated with the same batch of stripping film, exposed the same length of time at the same temperature (3–4° C.), and developed in one batch of developer simultaneously, and, finally, were stained simul-

taneously. This procedure was adopted to obtain more comparable results. It has been found that it is advisable to aim at a small number of disintegrations in the cells during the exposure time in order to obtain a low enough background for reliable grain counting. Scatter and cross fire will increase the background with increased exposure times or high concentrations of the isotope used. The general background on the autoradiographs was considerably less than 1 grain per 100 μ^2.

Differential counts and grain counts were performed on the stained preparations and the percentage of nuclei showing an autoradiograph was recorded. Since non-dividing cells do not synthesise DNA only cell types capable of division were counted, i.e., blasts, promyelocytes and myelocytes, and pronormoblasts, basophilic and early polychromatic normoblasts. However, the acid hydrolysis removes all cytoplasmic basophilia, thus the classification of the cells was based on the size, shape and structure of the nucleus. Later forms (e. g., metamyelocytes) incapable of division could not be excluded from such nuclear counts, and therefore there would be some cells counted which had had no DNA synthesis, and it is noted that in none of the counts were 100 per cent positive nuclei found.

The slides were code numbered prior to counting to reduce subconscious bias at the counting. On each slide 100-200 nuclei were counted, and grains were counted over-5-10 showing the strongest autoradiographs. This was to obtain the values for the maximum degree of DNA synthesis and was done after surveying over 500 nuclei. Repeated counts at different times were performed to test the reproducibility of the counts, and it was found that with the above technique the same observer showed an error not greater than $\pm$ 10–15 per cent.

For the calculations on the quantity of isotope incorporated into the cell, it was assumed (1) that the geometry of the autoradiographs was such that only 50 per cent of the electrons emitted from the disintegrations reached the photographic emulsion, and (2) that the ^{32}P electrons produced 0·7 grains per incident electron, and the ^{14}C electrons 2 grains per incident electron (Lamerton, 1950). If the exposure time and the half life of the isotope in question as well as the grain count per nucleus and the grain yield per electron of the isotope are known, then the number of the atoms of the isotope within the nucleus can be calculated. The technique is sensitive enough to detect 20–30 ^{32}P atoms per cell. Because of the very long half-life of ^{14}C (and because exposure times were limited to the relatively short period of 14–30 days) the minimum necessary number of ^{14}C atoms per nucleus to show an autoradiograph is of the order of 10^6.

In preliminary experiments the maximum DNA ^{32}P uptake per nucleus was found to be the order of 600 atoms (> 100 grains in 14 days' exposure 0·7 grains/electron). The DNA phosphorus content of the bone marrow cells has been found to be of the order of $8{\cdot}7 \times 10^{-7}$ μg (Davidson Leslie and White 1951*a*, 1951*b*), i.e., $1{\cdot}7 \times 10^{10}$ DNA P atoms/nucleus. The labelling obtained *in vitro* of the DNA is therefore of the order of $1 : 3 \times 10^7$. If the cells cannot differentiate between ^{31}P and ^{32}P then the total phosphorus pool available for the cells must also have been labelled in the ratio of the order of $1 : 3 \times 10^7$.

Thus for 5 μc. per ml. medium ^{32}P in 2 ml. medium $= 6{\cdot}7 \times 10^{11}$ ^{32}P atoms ($3{\cdot}6 \times 10^{-5}$ μg.)—the total pool should be $6{\cdot}7 \times 10^{11} \times 3 \times 10^7 = 2 \times 10^{19}$ atoms, i.e., approx. 1 *mg*. This figure in fact is probably an overestimate, since some DNA P will be lost during acid hydrolysis and the true labelling therefore will be higher than the figure $1 : 3 \times 10^7$ given above.

In actual fact the phosphorus pool was made up of

2 ml. serum culture medium (15 mg. per cent serum total P) = 0·3 mg.
0·1 mg./ml carrier phosphate buffer in 0·1 ml. ^{32}P solution = 0·01 mg.
total P in 10^7 cells/culture bottle = approx. (0·009 mg. DNA P) × 5 for total P = approx. 0·05 mg.
TOTAL POOL = < 0·4 mg.

This figure is less than half the value calculated from the experimental observations, but, considering the nature of these observations, the difference cannot be taken to indicate a real differentiation between ^{31}P and ^{32}P by the cells.

Adenine is not normally present in serum. The addition of adenine ^{14}C into the culture medium does not represent the labelling of a pool, but the provision of adenine to the cells which would otherwise have to synthesise it. The marked ability of the cells to take up adenine from the culture medium is shown by the fact that the average maximum uptake per nucleus was of the order of $1·2 \times 10^7$ atoms DNA ^{14}C (50 grains in 10 days' exposure, 2 grains/electron). The radioactive carbon administered as adenine-8-^{14}C can label both adenine and guanine in DNA. From the data on DNA P above the total amount of adenine + guanine per nucleus has been calculated to be of the order of $8·5 \times 10^9$ molecules—therefore the uptake of $1·2 \times 10^7$ atoms ^{14}C means a 1 : 700 labelling of the DNA purines. Since, however, the specific activity of the labelled adenine was 10 μc/mg. (i.e., 1 : 40 labelled) this means a 1 : 18 effective labelling of the DNA purines. This should be compared with the $1 : 3 \times 10^7$ labelling of the DNA obtained with ^{32}P, and explains why satisfactory autoradiographs can be obtained with ^{14}C with a half-life of over 5000 years.

The concentration of the adenine ^{14}C in the culture medium was 0·5 μc./ml. The culture bottles contained 2 ml. medium each, i.e., the number of adenine ^{14}C molecules/culture was of the order of 8×10^{15}. Therefore the total number of adenine sulphate molecules in the culture (for a 1 : 40 labelling in the sample, was of the order of $3·2 \times 10^{17}$ (50 μg./ml.), i.e., 0·26 milimolar. This concentration of adenine was found to be completely non toxic for the cells. If all the cells in the culture (10^7) would take up the maximum amount of adenine ^{14}C the total DNA uptake would be of the order of $1·2 \times 10^{14}$ atoms DNA ^{14}C, i.e., about 1/65 of the amount supplied in the culture medium.

RESULTS.

A. *The Cell Cycle.*

If the cells synthesised DNA throughout the entire intermitotic period, then, after a few hours of culture in the presence of the isotope all the cells should contain labelled DNA. This, however, was found not to be the case.

Tables I and II, and Fig. 1 show that when cells are cultured for progressively greater lengths of time, a gradually increasing percentage of the cells will show DNA autoradiographs (per cent positive nuclei), and the grain count (upper average grain count per nucleus) demonstrates that the amount of labelled DNA per nucleus also increases with time until reaching a maximum value. This indicates that DNA synthesis takes place during a limited period in the cell cycle.

It may be assumed that some time (A) must be spent by a cell in DNA synthesis before sufficient labelling to show an autoradiograph is obtained. There-

fore the cells within A hours of the end of the period of DNA synthesis at the start of the culture in isotope will not be labelled, and, similarly, cells entering the period of DNA synthesis during the culture time (T) and spending less than A hours

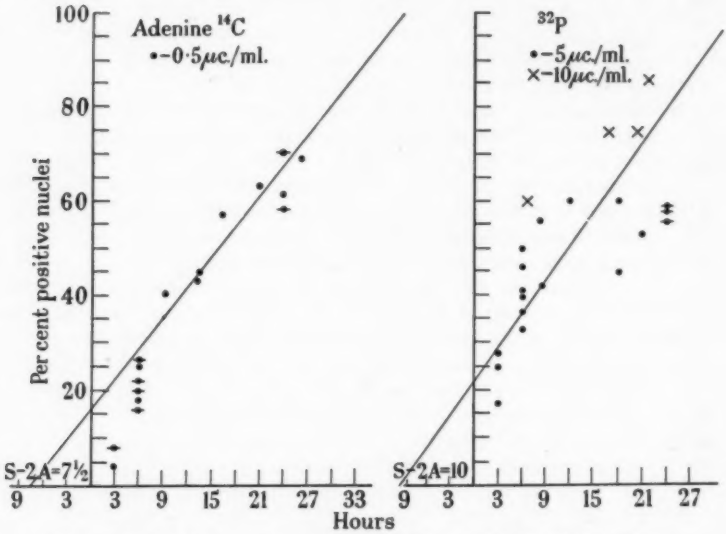


FIG. 1.—Rate of incorporation of adenine ^{14}C and ^{32}P into DNA *in vitro*.

TABLE I.—*The Rate of Incorporation of Adenine ^{14}C into DNA* in vitro

Exp. No.	Culture in isotope.	% positive nuclei.	Av. grains/nuclei.
191	0–3	4	8
193	0–3	8	15
197	0–3	8	<10
188	0–6	(a) 25	—
		(b) 26	—
191	0–6	(a) 18	—
		(b) 16	—
193	0–6	20	>15
196	0–6	(a) 22	15
		(b) 20	15
197	0–6	16	>10
199	0–6	26	15
199	18–24	22	15
184	0–9	40	—
184	0–13	(a) 43	—
		(b) 45	—
179	8–24	57	—
180	3–24	63	—
175	0–24	58	—
181	0–24	70	—
189	0–24	61	—
198	0–24	70	45
199	0–24	58	>30
185	0–26	69	—

TABLE II.—*The Rate of Incorporation of ^{32}P Orthophosphate into DNA* in vitro.

Exp. No.	Culture in isotope.	% positive nuclei.	Av. grains/nuclei.
182	0–3	28	—
192	0–3	17	30
192	21–24	25	30
171	0–6 (10 μc.)	(60)	—
192	0–6	40	>40
181	0–6	46	—
182	0–6	41	—
185	0–6	33	—
188	0–6	50	—
180	3–9	37	—
187	18–26	56	—
181	20–28	42	—
192	0–12	60	>60
160	0–18	45	—
179	8–24 (10 μc.)	(75)	—
177	4–24 (10 μc.)	(75)	—
178	3–24 (10 μc.)	(86)	—
196	6–24	60	>80
180	3–24	53	—
192	0–24	59	>80
169	0–24	58	—
196	0–24	59	100
185	0–26	56	—
198	0–24	58	>80
189	0–24	56	

in the period of DNA synthesis will not be labelled. Thus for any culture time (T) greater than this interval A, if S is the number of hours of the period of DNA synthesis, cells within a period of cell cycle given by $S + T - 2A$ will be labelled. For ^{32}P orthophosphate A is approximately $1\frac{1}{2}$ hours, for adenine ^{14}C 3 hours.

The period $S + T - 2A$ will be proportional to the percentage of cells labelled and a plot of T against per cent labelling should give a straight line. Zero labelling will correspond to a value of $T = -(S-2A)$, i.e., if the straight line is produced back to cut the T axis at some value $-T_1$ hours, then the length of the period of DNA synthesis in hours (S) will be given by $T_1 + 2A$. 100 per cent labelling will occur when cells which were just at the end of the period of DNA synthesis have gone through the whole cycle and entered DNA synthesis again for A hours to obtain detectable labelling. If the time of culture for 100 per cent labelling is T_2, the total cell cycle time C will be given by $T_2 + S - A$ hours. In practice the time for 100 per cent labelling will be obtained by producing the straight line to this value.

Series of cultures both with adenine ^{14}C and with ^{32}P labelling of the DNA indicate a length of period of DNA synthesis (S period) of the order of 12–15 hours, and a total cell cycle time of about 40–45 hours. The length of the S period is also indicated by the gradual increase of the grain count/nuclei up to about 15 hours, then reaching the maximum average value for a given concentration of the isotope in the culture medium. The length of the total cell cycle can also be calculated in a different way. A 6 hours' culture in adenine ^{14}C or a 3 hours' culture in ^{32}P labels only the cells already in the S period ($A = 3$ hours for adenine ^{14}C, $1\frac{1}{2}$ hours for ^{32}P; and number of cells entering S period in $1\frac{1}{2}$ to 3 hours is relatively small). As can be seen from Fig. 1, a 6 hours' culture in adenine

^{14}C or a 3 hours' culture in ^{32}P labels slightly less than 25 per cent of the cells. If, then, in a cell population randomly distributed over the cell cycle, at any given time one-quarter of the cells are in the *S* period, then the total cycle must last four times that period, i.e., over 48 hours in the case of a 12 hours' *S* period.

When only the mitotic figures are counted on the smears (metaphase and anaphase) it appears that while in a 2–4 hours' culture with ^{32}P the majority of the mitotic figures do not show autoradiographs, in a 6 hours culture most of the mitoses already contain labelled DNA (Table III and Fig. 2). This observation

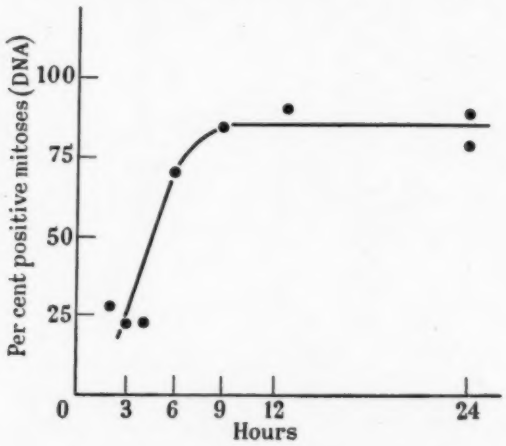


FIG. 2.—Demonstration of the "G_2" period.

TABLE III.—*Demonstration of the G_2 Period in the Cell Cycle when no Appreciable DNA Synthesis Takes Place.*

(Measured with ^{32}P or adenine ^{14}C incorporation into DNA.)

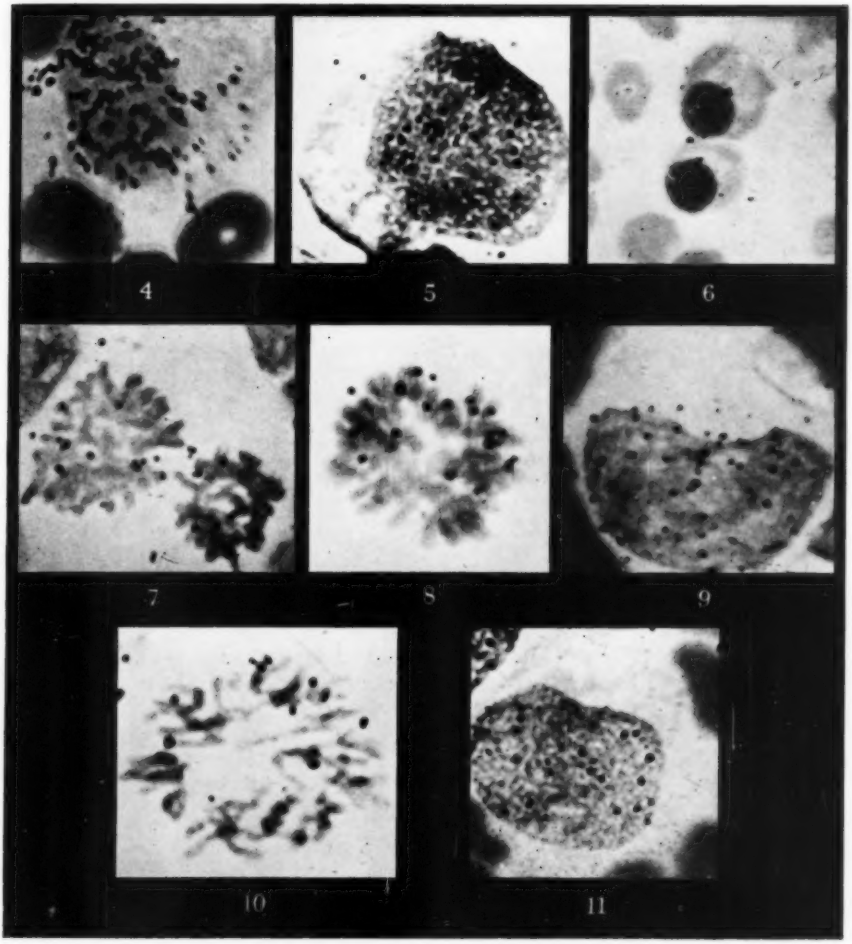
Exp. No.	Isotope.	Culture in isotope.	% positive mitoses.	Av. grains/ mitoses.	Av. grains/ resting nuclei.
203	^{32}P	10–12	27	<15	>35
		9–12	24	<15	>35
		8–12	24	<15	>35
181	^{32}P	0–6	70	—	—
184	adenine ^{14}C	0–9	86	—	—
184	" ^{14}C	0–13	90	—	—
181	" ^{14}C	0–24	88	—	—
174	" ^{14}C	0–24	78	—	—

EXPLANATION OF PLATES.

FIG. 4–6.—Adenine ^{14}C uptake by human bone marrow cells *in vitro*. Smears fixed in alcohol: stained autoradiographs. Note the mainly nuclear localization of the isotope (in RNA and DNA).
(1) myelocyte, × 1500; (2) pronormoblast, × 1500; (3) plasmacytes, × 750.

FIG. 7–9.—Adenine ^{14}C uptake into DNA by human bone marrow cells *in vitro*. Smears fixed in alcohol, then hydrolysed in N HCl at 60° C. for 6½ minutes. Stained autoradiographs, × 1500.

FIG. 10, 11.—^{32}P uptake into DNA by human bone marrow cells *in vitro*. Smears fixed in alcohol. then hydrolyzed in N HCl at 60° C. for 6½ minutes. Stained autoradiographs, × 1500.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suggests that immediately prior to mitosis there is a 3–4 hours' stage of the cell cycle (G_2) during which no DNA synthesis takes place Prior to this second gap (G_2) is the period of DNA synthesis (S), which is preceded by the long first gap (G_1) during which again no DNA synthesis takes place. Fig. 3 illustrates the timing of the cell cycle obtained with the methods outlined above. This timing, in principle agrees well with that found in the bean root by Howard and Pelc (1951).

It can be seen from Tables I and II and Fig. 1 that although DNA labelling with both isotopes gives essentially the same results, the values for adenine ^{14}C are more consistent than those obtained with ^{32}P. The reason for this difference is, firstly the differences between energy of the beta particles emitted by ^{14}C and ^{32}P. The higher energy ^{32}P electrons result in a greater scatter and, consequently, in a higher background, thus interfering with the accuracy of the counts. Secondly, while the half life of ^{14}C is a negligible problem when the isotope dilutions are added to the culture medium, variations in volume to allow for the decay are a source of error with ^{32}P. Also, due to the decay of ^{32}P and to the fact that ^{32}P is supplied in ^{31}P phosphate buffer, the ratio of ^{31} to ^{32}P will not be constant: the degree of labelling of the phosphorus pool will be variable. These factors all contribute to the lesser accuracy of the counts in cultures with ^{32}P labelling as compared with the ^{14}C labelling.

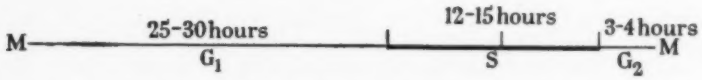

FIG. 3.—The cell cycle related to DNA synthesis, measured by the incorporation of ^{32}P or adenine ^{14}C into DNA.

In some experiments with ^{32}P orthophosphate a concentration of 10 μc./ml. medium was used instead of the usual 5 μc./ml. In short time cultures (3–6 hours) where the average grain count per nucleus is low, the higher isotope concentration results in an undesirable increase in the background. In cultures of 12 hours or over, however, the higher labelling of the pool increases the grain count per nucleus considerably and more reliable counts can be obtained than with the lower isotope concentration which labels some cells only slightly above the background of the long term cultures.

B. *Rate of DNA Synthesis in Different Types of Bone Marrow Cells.*

In these experiments only those types of cells were found to synthesise DNA which are known to be capable of mitosis. No differences were found between the same types of cells whether they originated in normal or leukaemic marrows.

The cell cycle as illustrated in Fig. 3 represents the average values for the majority of the dividing cells of the bone marrow, i.e., promyelocytes, myelocytes, pronormoblasts, basophilic normoblasts and early polychromatic normoblasts. In a few experiments, however, where it was found to be possible to count early normoblasts and early myeloid cells separately, a shorter cycle than in Fig. 3 was indicated for the normoblasts. Blast cells from acute leukaemia have shown a strikingly slower rate of DNA synthesis than the promyelocytes and myelocytes

of the normal marrow or myelocytic leukaemia marrow (Table IV). No difference was found in this respect between normal or leukaemic serum as culture medium. This slow growth rate of the blast calls as opposed to the more mature forms was

TABLE IV.—*Comparison of the Rate of Adenine ^{14}C Uptake into DNA by Normal Bone Marrow Cells and Blast Cells from Leukaemia.*

Type of marrow.	Culture in isotope (hr.).	% positive nuclei.	Av. grains/ nuclei.	Remarks.
6 normal marrows	0–6	21 (mean)	15	(Normal serum)
Blast cell leuk. No. 194	0–6	7	6	,, ,,
		5	5	(leuk. serum)
Blast cell leuk. No. 183	0–6	1	4	(Normal serum)
5 normal marrows	0–24	65 (mean)	>30	,, ,,
Blast cell leuk. No. 194	0–24	20	<25	,, ,,
		20	<25	(leuk. serum)

surprising in view of the rapid clinical course of the blast cell leukaemias: on the other hand it is compatible with the low mitotic index usually found in blast cell leukaemia marrows. Blast cells in normal marrows are too scanty to allow quantitative assessment of their cycle time, but the observations in the cultures suggested a similar cycle time to that obtained with leukaemic blast cells.

No appreciable DNA synthesis was detected in the lymphocytes whether normal or from lymphocytic leukaemia. Similarly, no appreciable DNA synthesis was detected in the plasmacytes from normal marrows. Plasmacytes from one case of multiple myeloma showed a limited amount of DNA synthesis, less than found in blast cells.

With increase in the degree of maturation the cells lose the ability to synthesise DNA. Thus no DNA synthesis was detected after the metamyelocyte stage, or the late polychromatic normoblast stage.

C. *RNA Turnover.*

Smears not hydrolysed with N HCl, or even better, smears treated with the enzyme desoxyribonuclease give a picture of the ribose nucleic acid (RNA) turnover in the cells. It was found that the strongest uptake of RNA ^{32}P or RNA adenine ^{14}C is in the nucleus. The cytoplasm of the early normoblasts and promyelocytes has also shown uptake, but never to the same extent as the nucleus (Fig. 4–11). The very slight cytoplasmic uptake of ^{32}P or adenine ^{14}C into RNA of the plasma cells was surprising. Both plasma cells and lymphocytes show a good protein turnover as measured with methionine ^{35}S uptake. It seems that an active protein turnover is not necessarily accompanied by an active RNA turnover.

D. *The Effect of X-ray Irradiation on DNA Synthesis*

Large doses of X-rays (5000 r in 15 minutes, 140 kV, l./mm. Al filter) exerted a marked inhibition on DNA synthesis. As is shown by Tables V and VI and Fig. 12, if in a 0–6 hour culture in isotope, radiation is delivered in the 3rd hour

TABLE V.—*Effect of 5000 r on the Incorporation of Adenine ^{14}C into DNA.*

(Radiosensitivity of the "S" period.)

Exp. No.	Culture in isotope.	% positive nuclei.	Av. grains/nucleus.
193	0–3	8	8
197	0–3	8	10
193	0–6	20	>15
197	0–6	16	>10
199	0–6	26	15
193	0X–6	1	< 5
197	0X–6	1	5
193	0–3X–6	8	<8
197	0–3X–6	5	>5
199	0–3X–6	6	<10

TABLE VI.—*Effect of 5000 r on the Incorporation of ^{32}P into DNA.*

(Radiosensitivity of the "S" period.)

Exp. No.	Culture in isotope.	% positive nuclei.	Av. grains/nuclei.
182	0–3	28	—
192	0–3	17	30
181	0–6	46	—
182	0–6	41	—
185	0–6	33	—
192	0–6	40	>40
180	3–9	37	—
*181	0X–6	14	—
192	0X–6	16	10
182	0–3X–6	26	—
185	0–3X–6	18	—
192	0–3X–6	33	30

((0–3X–6) then both the number of cells showing DNA autoradiographs and the grain count over the nuclei give similar counts to those in a 3 hour culture (0–3). This observation suggests that cells in the period of DNA synthesis are immediately and completely prevented from synthesising DNA. Radiation

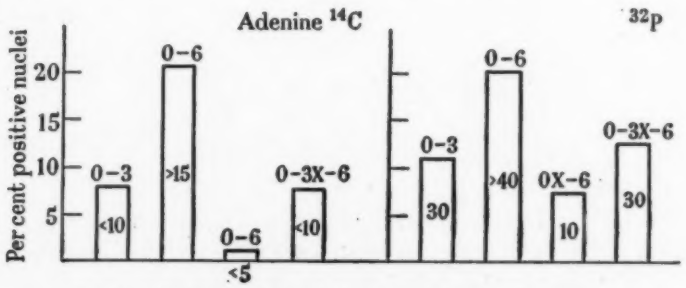

FIG. 12.—Radiosensitivity of the "*S*" period. × = 5000r.

delivered at the beginning of the culture period in isotope (0X–6) also markedly inhibits DNA synthesis. The low grain counts and the low numbers of cells showing DNA autoradiograph suggested that it was probably the arrival of cells from the G_1 period into the S period during the culture which gave the autoradiographs. However, if cells in the G_1 period had not been damaged by the radiation, then those at the end of the G_1 period at the time of irradiation should have entered the S period and should have synthesised that amount of DNA normally synthesised in 6 hours. Since the grain counts indicated that subsequent to irradiation no cells, even with long culture times, synthesised more DNA than a 2–3 hour non-irradiated culture would (Fig. 12 and 13), it was suspected that the cells in the G_1 period suffered a latent damage.

Tables VII and VIII and Fig. 13 show that if the isotope was added to the cultures 3 or more hours after irradiation (X–3–24) hardly any DNA synthesis was observed, as opposed to those cultures in which the isotope was added imme-

TABLE VII.—*Effect of* 5000 *r on the Incorporation of* ^{14}C *Adenine into DNA*

(Demonstration of the " latent damage.")

Exp. No.	Culture in isotope.	% positive nuclei.	Av. grains/nuclei.
181	0–24	70	—
189	0–24	61	—
198	0–24	70	45
199	0–24	58	>30
185	0–26	61	—
180	3–24	63	—
199	3–24	58	>30
198	4–24	70	45
189	0X–24	12	—
198	0X–24	25	20
199	0X–24	20	15
*180	X–3–24	14	—
199	X–3–24	10	6
198	X–4–24	5	10

* Due to mistake in filtration the dose of irradiation in this culture was probably considerably less than 5000 r.

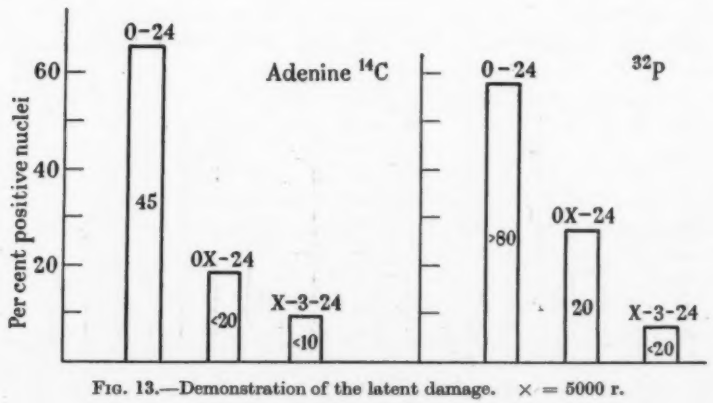

FIG. 13.—Demonstration of the latent damage. × = 5000 r.

TABLE VIII.—*Effect of* 5000 *r on the Incorporation of* ^{32}P *into DNA.*

(Demonstration of the " latent damage.")

Exp. No.	Culture in isotope.	% positive nuclei.	Av. grains/nuclei.
169	0–24	58	—
185	0–26	56	—
189	0–24	56	—
192	0–24	59	>80
196	0–24	59	100
198	0–24	58	>80
180	3–24	53	—
196	6–24	60	>80
189	0X–24	30	—
192	0X–24	30	15
196	0X–24	21	>20
196	X–3–24	9	<20
196	X–6–24	7	20

diately following the irradiation (0X–24). These observations suggest that cells in the G_1 period receive a latent damage from a dose of 5000 r. The latent period is about 3 hours during which the cells may enter the S period (if at the time of irradiation they were near the end of G_1 period). After about 3 hours, however, even these cells will be incapable of DNA synthesis.

The comparison of cultures incubated for 6 hours after irradiation (0X–6) and for 24 hours after irradiation (0X–24) indicated a certain amount of " post-irradiation recovery " (Fig. 14). The grain counts indicated, however, that this

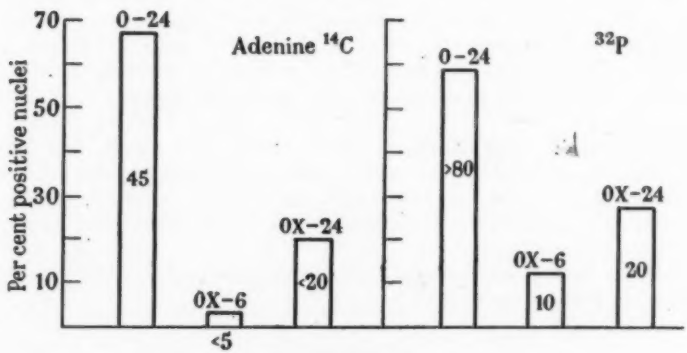

FIG. 14.—Post-irradiation DNA synthesis.

was not a recovery in the sense of ability to synthesise normal amounts of DNA, but the entry of some G_1 cells into the S period for a limited time. It appears that all G_1 cells are damaged by a dose of 5000 r.

The effect of irradiation on the G_2 period is indicated by the fact that no mitoses were observed in any of the irradiated cultures.

A dose of 5000 r produced a marked degeneration of the cells in the cultures, but over 6 hour culture times were needed to show the first morphological signs

of post-irradiation degeneration. The complete inhibition of mitoses was, however, immediate.

In agreement with previous findings by other workers it was found that even this large dose of radiation produced only a moderate decrease in RNA turnover. This should be compared with the effect of barbiturate (Nembutal Abbot 0·5 mg./ml. medium) which not only markedly inhibited DNA synthesis, but also markedly inhibited RNA turnover in the cells (Table IX).

TABLE IX.—*Effect on Nembutal on Adenine ^{14}C Uptake.*

Type in culture.	Culture in isotope. (hr.).	DNA		RNA	
		%+ nuclei.	Av. grs./ nuclei.	%+ nuclei.	Av. grs./ nuclei.
Control . . .	0–24 .	70	45	90	80
Nembutal 0·5 mg./l. .	0–24 .	11	10	90	20

DISCUSSION.

DNA synthesis was defined above as the incorporation of ^{32}P or adenine ^{14}C into DNA. In a sense this could be better defined as the assembly of the DNA molecule. Experiments with the synthesis of the purines and pyrimidines from labelled one carbon compound precursors (*e.g.*, formate ^{14}C) are in progress.

The timing of the cell cycle is, in principle, in good agreement with that described in the bean root cell by Howard and Pelc (1951). It is expected that the timing of the cell cycle in different mammalian tissues will yield different lengths of cycles. It remains to be seen whether in longer or shorter cycles than illustrated above, all periods are longer or shorter, or only G_1 (and possibly G_2). It would not be surprising to find that DNA synthesis is such a rigidly standardised process in Nature that the synthesis of a given diploid amount always takes the same time.

The large dose of radiation employed in these experiments was deliberately chosen. Experiments with lower doses and with varying dose rates are in progress, and it may be that the response of the cells under such conditions will be qualitatively as well as quantitatively different from those described in this paper.

This work is part of an investigation carried out under a full grant from the British Empire Cancer Campaign.

SUMMARY.

(1) Using the incorporation of ^{32}P and adenine ^{14}C into DNA as indicators of DNA synthesis it was possible to study and to time the period of DNA synthesis in the intermitotic cycle of human bone marrow cells *in vitro*. The total cycle time was found to be of the order of 40–48 hours for the average dividing bone marrow cells (promyelocytes, myelocytes, pronormoblasts, basophilic and polychromatic normoblasts). The period of DNA synthesis was found to be of the order of 12–15 hours taking place in the second half of the cycle, and divided from the mitosis by a 3–4 hours non-synthesising period.

(2) Only cell types capable of division showed DNA synthesis.

(3) No difference was found between the same types of cells whether originating in normal marrows or in marrows of patients with leukaemia.

(4) Blast cells from acute leukaemias showed a markedly slower rate of DNA synthesis (growth rate) than promyelocytes and myelocytes from normal or leukaemic marrows.

(5) The early nucleated red cell series (pronormoblasts and basophilic normoblasts) showed a slightly faster rate of DNA synthesis than the promyelocytes and myelocytes.

(6) No appreciable DNA synthesis was observed in the lymphocytes whether normal or leukaemic.

(7) 5000 r X-ray irradiation inhibited DNA synthesis immediately and completely.

(8) Cells before the period of DNA synthesis developed a latent damage after 5000 r which either completely abolished or considerably decreased their ability to synthesise DNA.

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CORRELATION OF SPECTRAL SHIFTS AND CARCINOGENIC POWER

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MANY attempts have been made to relate the carcinogenic activity of aromatic hydrocarbons to measured and calculated physical properties with very little success. Ease of photoxidation (Cook and Martin, 1940), electronic spectra (Miller and Baumann, 1943), polarizability, free valence at a particular atom, bond order of a particular bond (in so-called K region), (Pullman, 1947), self-polarizability (Greenwood, 1951) are typical examples.

We have examined the visible and ultra-violet spectra in absorption and in emission of a large number of closely related carcinogens and have attempted to correlate them with carcinogenic power. We have also examined various spectral properties depending on the degree of interaction of the carcinogen with other molecules in its vicinity, since the initial step in the chain of events resulting in carcinogenesis must involve some such interaction.

EXPERIMENTAL.

Precise experimental details are given elsewhere (Lewis and Kasha, 1944; Moodie and Reid, 1952). The emission spectra which yielded most results of interest were obtained by dissolving (or suspending, in the case of the heterogeneous systems examined) the carcinogen in an inert aliphatic medium which could be frozen to a clear transparent glass at the temperature of liquid nitrogen (−190° C.). The samples were frozen in cylindrical tubes immersed in a quartz Dewar vessel and were irradiated with filtered light from a high pressure mercury arc, either the 3100 Å or the 3650 Å group of lines being used. Under these conditions all the molecules examined were strongly luminescent, showing both a short-lived fluorescence (persisting for less than 10^{-8} seconds) and long-lived phosphorescence (persisting for several seconds) in different spectral regions. Fluorescence spectra were traced photoelectrically using a Hilger E_2 spectrograph equipped with a Scanning Unit. Phosphorescence spectra were photographed using a mechanical phosphorescope as described elsewhere (Lewis and Kasha, 1944; Moodie and Reid, 1952). Both solvents and carcinogens were purified by repeated distillation and chromatography where necessary.

RESULTS AND DISCUSSION.

(1) No significant correlation could be found between absorption or short-lived spectra and carcinogenic activity, and these spectra, which have already been considered by other workers, will not be discussed. However, some correlations

exist between the position of the phosphorescence bands, which lie for the most part in the red region of the spectrum, and carcinogenic power. The results are shown in Table I. In the set of 15 substituted 1,2-benzanthracenes, most of the strong carcinogens have phosphorescence band origins at or below 16,000 cm^{-1} (i.e., above 610 millimicrons) while, in general, the origins of the weak carcinogens' phosphorescence bands lie above 16,400 $cm.^{-1}$. Similarly in the series of 3, 4-benzophenanthrene* the strong carcinogens have phosphorescence band origins below 19,800 $cm.^{-1}$ (i. e., above 505 millimicrons) while the weak carcinogen's phosphorescence has a higher energy.

TABLE I.—*Positions of Emission Band Maxima for the Long-lived Phosphorescence Bands of Some Hydrocarbons and Carcinogenic Power.*

Hydrocarbon.	Activity.	Origin of phosphorescence band system $cm.^{-1}$.
1, 2-benzanthracene	0	16,500
9, 10-dimethyl-1, 2-benzanthracene	++++	15,500
10-ethyl-1, 2-benzanthracene	+++	16,370
1′methyl-1, 2-benzanthracene	0	16,980
2′ " " "	0	16,640
3′ " " "	0	16,720
4′ " " "	0	16,670
3-methyl-1, 2-benzanthracene	+	16,810
4 " " "	+	16,980
5 " " "	++	16,650
6 " " "	+	16,530
7 " " "	+	16,580
8 " " "	+	16,780
9 " " "	++	16,260
10 " " "	+++	16,310
3, 4-benzophenanthrene	+	19,840
1-methyl-3, 4-benzophenanthrene	Unknown	18,940
2 " " "	++	19,760
3 " " "	++	19,760
4 " " "	++	19,720
5 " " "	+++	19,720
6 " " "	+++	18,690

This result may be a significant one. The phosphorescence spectra are the result of excitation to the very lowest excited state of the carcinogen molecule, a " triplet " state resulting from the fact that there are two unpaired electrons. Excitation of this state requires only about 45 k.cal., much less energy than is necessary to break bonds in the molecule, and its formation may well be an intermediate step in the reaction with protein. In this connection it should be pointed out that the triplet levels, since they cannot be detected in absorption, the common method of observing spectra, have been much neglected by biologists in their considerations of low barrier reaction paths that may be involved in biochemical reactions.

(2) The second significant correlation comes from heterogeneous system experiments. When suitable pairs of molecules are incorporated in the same system the fluorescence spectrum of the resulting mixture often shows changes due to interaction between the two components (Lewis and Kasha, 1944; Moodie and

* 3, 4-benzophenanthrene is the compound called benzo (c) phenanthrene in *Chemical Abstracts* (U.S.A.).

Reid, 1952). In the case of pairs of aromatic hydrocarbons the interaction between two such substances in true solution is very weak indeed at the low concentrations necessary for low temperature fluorescence experiments. Much stronger interactions are often observed if either one of the components is in the form of a microcrystalline suspension while the other is in true solution. Most of the emitted light then appears to come from the surface of the crystals where a layer of the dissolved component is adsorbed. We have found that if a carcinogen is used as the microcrystalline component and the dissolved component is naphthacene, the (fluorescent) emission of the naphthacene is shifted slightly in wave length because of its interaction with the carcinogen. Some of the results are shown in Table II. It will be observed that the smallest shifts in the naphthacene bands occur when the strongest carcinogens are used as the host crystal. There is a moderately good correlation between these shifts and the self-polarizabilities at the position of the substituent calculated by Greenwood (1951), which values themselves correlate better with carcinogenic power than do any other physical constants. The explanation for the observed phenomena must be that the more polarizable the host crystal the more the adsorption process is accompanied by distortion of its orbitals, leaving the orbitals of the adsorbed naphthacene unchanged. Conversely if the host crystal molecule is not polarizable the naphthacene is considerably polarized in the crystal field with corresponding shift of its electronic energy levels.

TABLE II.—*The Shift from its Value in Solution of the Naphthacene Emission Band at −190° C. in Suspensions of Various Hydrocarbons compared with the Carcinogenic Activity (Pullman, 1947) and Self-polarizability Values (Greenwood, 1951), at the Substituent Position.*

Component in suspension.	Naphthacene solution band shift.	Activity.	Self-polarizability.
1, 2-benzanthracene	603 cm.$^{-1}$	0	—
9, 10-dimethyl-1, 2-benzanthracene	306 „	++++	—
10-ethyl-1, 2-benzanthracene	392 „	+++	—
1′ methyl-1, 2-benzanthracene	561 „	0	0·439
2′ „ „ „	645 „	0	0·410
3′ „ „ „	634 „	0	0·404
4′ „ „ „	498 „	0	0·439
3 „ „ „	624 „	+	0·448
4 „ „ „	392 „	+	0·447
5 „ „ „	392 „	++	0·452
6 „ „ „	645 „	+	0·409
7 „ „ „	686 „	+	0·410
8 „ „ „	498 „	+	0·449
9 „ „ „	561 „	++	0·496
10 „ „ „	434 „	+++	0·514

For molecules other than the 1,2-benzanthracenes self-polarizabilities were not available to us, but the correlations with biological activity were less convincing.

SUMMARY.

(1) Within a series of carcinogens with the same basic ring structure carcinogenic power correlates with position of the lowest triplet level (phosphorescence emission spectrum).

(2) Spectral shifts in complexes between naphthacene and carcinogens also correlate well with carcinogenic power.

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ALKALINE PHOSPHATASE ACTIVITY IN FOUR TRANSPLANTABLE MAMMARY CARCINOMAS OF MICE

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In a comparative study of alkaline phosphatase in tumors, Greenstein (1942) noted that the mouse tumours studied possessed little or no alkaline phosphatase activity with the exception of spontaneous mammary tumors and lymphomas. Comparative Q values were calculated as the ratio of the percentage of disodium-phenyl phosphate hydrolyzed to milligrams of total N per cubic centimeters of tissue extract. Several concentrations of N were taken and the Q values remained remarkably constant in relation to nitrogen. By this means a parameter was established which could be utilized for the comparison of the mouse tumors thus tested. In the mouse the mammary tumor had the highest Q value. Both tumors tested were the spontaneous mammary tumor in C3H mice with a Q value of 22 and spontaneous mammary tumor in A mice with a Q value of 20. The highest value for any tumor was the transplanted hepatoma 31 in rats with a Q value of 542.

Kabat and Furth (1941) could not demonstrate alkaline phosphatase by means of Gomori procedure in mammary adenocarcinoma spontaneously arising in C3H stock. However, Hard, Pratt-Thomas and Belkin (1948) examined twenty-nine spontaneous mammary carcinomas in C3H, A and DBA mice and noted high alkaline phosphatase activity. The predominent picture in the mouse tumours examined was the high alkaline phosphatase activity shown by the cells of the acinar units while the stroma and vascular elements were free. They pointed out two consistent variations, the first occurred in areas in which the cancer cells were arranged in alveolar units, and second in anaplastic areas of the tumor. In the first instance the cells of the alveolar units were free from alkaline phosphatase whereas the stroma showed an intense reaction. In the second instance it was thought that the more anaplastic areas show decrease in enzyme activity.

In previous investigation from our laboratory on a different tumor by Pearson, Morrione and Novikoff (1949), (1950), it was shown for hepatomas in rats induced by *p*-dimethylaminoazobenzene that the previous high values of alkaline phosphatase were due in a large part to the proliferation of non-neoplastic bile ducts, necrotic tissue, and vascular sprouts accompanying the growing tumor.

In his extensive work on tumors, Greenstein (1947) stated that tumors resemble each other more nearly chemically and enzymatically than normal tissues, whereas the variation in normal mouse tissue of alkaline phosphatase is wide, in tumors the range becames narrow except for osteogenic sarcoma (Greenstein, 1945).

The purpose of this investigation was to determine whether such uniformity existed and to seek a more definitive answer to the problem of the relationship of alkaline phosphatase to various types and strains of tumors.

MATERIALS AND METHODS.

The four transplantable mammary tumors used in this experiment were E0771, H2712, dbrB, and 15091a. In designating the strains used the standardized nomenclature recommended by Carter *et al.* (1952) was used.

The Tumor E0771 originated spontaneously in 1939 in the Roscoe B. Jackson Memorial Laboratory from the mammary gland in a C57 transplant generation. The animal giving rise to the original tumor was a F_3 descendent of a mouse that was born from a fertilized ovum transplanted into the uterus of a DBA mouse and was nursed by her DBA mother. This has gone through 320 transplant generations. Results from the Jackson Laboratory indicates 100 per cent transplantability in C57BL and their F_1 hybrids. The histological type is an adenocarcinoma (Snell, Cloudman and Woodworth, 1948).

The Tumor H2712 originated spontaneously in the mammary gland of a C3H mouse in 1948 at the Roscoe B. Jackson Memorial Laboratory. This has gone through 86 transplant generations and shows 100 per cent transplantability in C3H strains and their F_1 hybrids. The histological type is an adenocarcinoma.

The Tumor dbrB originated in the Department of Genetics, Carnegie Institute of Washington in 1918 in the mammary gland of a DBA/1 mouse and has gone through 845 transplant generations. Results from the Roscoe B. Jackson Memorial Laboratory show 100 per cent transplantability in DBA strains and their F_1 hybrids. The histological type is an adenocarcinoma (Strong and Little, 1920; Little and Strong, 1924).

The Tumor 15091a originated in the laboratory of the University of Michigan in the mammary gland of an A albino mouse in 1928 (Cloudman, 1928). This has gone through 341 transplant generations and shows 100 per cent transplantability in A albino and their F_1 hybrids. The histological type now is that of an anaplastic carcinoma.

For the purposes of the experiment the animals were divided into four series. Series I consisted of Tumor E0771 transplanted into males and females of C57BL and their F_1 hybrids between C57BL and A/L. Series II consisted of Tumor H2712 transplanted into C3H males and females. Series III consisted of Tumor dbrB transplanted into DBA male and female. Series IV consisted of Tumor 15091a transplanted into F_1 hybrids of C57BL and A/He male and female.

The total number of animals of the four series were 100 and 81. All mice were transplanted with their respective tumor at the Roscoe B. Jackson Memorial Laboratory and sacrificed at intervals between 7 and 22 days or an average of 16 days.

The animals were killed by ether anesthesia and thin slices of the liver, spleen, adrenals, kidney, testes, ovary and the tumor were placed immediately into formol-saline and into ice-cold acetone. Tissues placed in formol-saline were stained by the regular hemotoxylin and eosin methods. Tissues placed in ice-cold acetone were left for 24 hours with one change of acetone. They were then placed in cold absolute alcohol over night followed by two changes of benzol of 45 minutes each. They were then imbedded and infiltrated in paraffin at 56° C. for 2 hours, one-half of this time being in the vacuum oven. The resulting slides were run through xylol, absolute alcohol, 95 per cent alcohol and water.

Following this they were incubated at 37° C. in a substrate composed of 25 ml. 2 per cent sodium barbital, 25 ml 2 per cent calcium chloride, and 2 ml. 2 per cent magnesium sulphate. The solution was adjusted to a pH of 9·4.

After incubation the slides were rinsed in two changes of 1 per cent calcium chloride, and placed in 2 per cent cobalt nitrate for 2 minutes, distilled water for 2 minutes, dilute ammonium sulphide (10 drops per 100 c.c.) 2 minutes, and running tap water for 3 minutes. They were then dehydrated and mounted in Permount.

Incubation times in the substrate were as follows : Series I, II, III : 1 minute, 5 minutes, 15 minutes and 1 hour, and Series IV : 1 minute, 5 minutes, 15 minutes, 1 hour, 1½ hours, 2 hours and 2½ hours. A total of 1124 slides was prepared. All the slides were read and visually graded according to their intensities from 1 to 6. In this way a relative assay could be arrived at which showed a distribution within each incubation time, and the earliest reaction or end point could be ascertained. Controls were run by omitting the glycerophosphate in the substrate.

RESULTS.

Results of graded Gomori reactions.

Series I consisted of 39 transplanted E0771 mammary tumors. A total of 156 slides was examined in this series, representing a total of all the incubation times of a tumor. In addition, and in a similar way, other organs of the tumor-bearing host were examined. Table I represents the intensities plotted against the incubation time of each of the 39 slides in glycerophosphate substrate. At 1 minute incubation time 29 of the 39 reactions had a reaction intensity of one and hence this was considered as the "end point." At 5 minutes there were no negative reactions, the majority of reactions being two or three. At 15 minutes' incubation time the majority of reactions were three and four, and at 1 hour's incubation the reactions were intense.

TABLE I.—*Distribution of Graded Gomori Reactions in 39 Transplanted EO771 Mammary Carcinomas. (Series I.)*

Reaction intensities	1 min.	5 min.	15 min.	1 hour.
6				26
5			4	12
4			19	1
3		12	12	
2	1	19	4	
1	29	8		
0	9			

The overall alkaline phosphatase activity in this series was extremely intense. It was present mainly in the cytoplasm. The connective tissue and vessels of the stroma were entirely negative even at the 1 hour incubation time. Thus, none of the reaction could be attributed to the supporting stroma but was inherent in the tumor cells proper.

Series II consisted of 46 transplanted H2712 mammary carcinomas. A total of 184 slides were examined in this series representing the total incubation times of

the tumor. In addition, and in a similar way, other organs were examined. In this series the reaction intensities are illustrated in Table II. At 1 minutes' time 45 of the 46 reactions are negative. At 5 minutes' incubation time 32 of the 46 reactions are designated as one. Thus this incubation time was considered as the "end point." At 15 minutes the majority of the reactions are two and at 1 hour four.

TABLE II.—*Distribution of Graded Gomori Reactions in 46 Transplanted H2712 Mammary Carcinomas. (Series II.)*

Reaction intensities.	1 min.	5 min.	15 min.	1 hour.
6				
5				4
4				25
3			5	17
2		1	24	
1	1	32	17	
0	45	13		

The alkaline phosphatase reaction was similar to Series I in so far as the tumor cells and stroma was concerned. The distribution was, however, strikingly different from the other tumors and will be referred to in the subsequent section.

Series III consisted of 48 transplanted dbrB mammary carcinomas. A total of 192 slides were examined, representing the total incubation times of this series. Other organs were also examined in a similar manner. The distribution of the reaction intensities are shown in Table III. At 1 minute incubation time all 48 reactions were negative and at 5 minutes 46 of 48 reactions were negative. The "end point" appeared to be at 15 minutes incubation time as 47 of 48 reactions were of the order of one.

TABLE III.—*Distribution of Graded Gomori Reactions in 48 Transplanted dbrB Mammary Carcinomas. (Series III.)*

Reaction intensities.	1 min.	5 min.	15 min.	1 hour.
6				
5				12
4				27
3				7
2				2
1		2	47	
0	48	46	1	

Series IV consisted of 48 transplanted 15091a mammary carcinomas as shown in Table IV. A total of 336 slides was examined in this series. There was no reaction present in this series with the incubation times as indicated even up to 2½ hours. The tumor was examined for stroma but very little was present and this showed a negative reaction. The vessels throughout the tumor showed a positive reaction. Other organs were examined in a similar manner.

TABLE IV.—*Distribution of Graded Gomori Reactions in 48 Transplanted 15091a Mammary Carcinomas. (Series IV.)*

Reaction intensities.	1 min.	5 min.	15 min.	1 hr.	1½ h.	2 hr.	2½ hr.
6							
5							
4							
3							
2							
1							
0	48	48	48	48	48	48	48

EXPLANATION OF PLATES.

FIG. 1.—Tumor E0771 transplanted into C57BL and F_1 hydribs (Series I). Alkaline phosphatase reaction at 15 minutes' incubation time slides show irregular areas of activity. × 120.

FIG. 2.—Tumor 15091a transplanted into F_1 hybrids of C57BL (Series IV). No alkaline phosphatase reaction at 15 minutes' incubation time. × 120.

FIG. 3.—Tumor dbrB transplanted into DBA (Series III). Alkaline phosphatase reaction at 15 minutes' incubation time shows a tendency of the peripheral cells of islands to show more activity than central portion. The supporting connective tissue shows no reaction. × 120.

FIG. 4.—Same tumor and series as Fig. 1, except incubation time is 1 hour. The marked intensity of the alkaline phosphatase reaction obscures detail. × 120.

FIG. 5.—Same tumor and series as that in Fig. 2 except the incubation time is 1 hour. The alkaline phosphatase reaction is still negative at this incubation time. Note the positive reacting vessels in the tumor. × 120.

FIG. 6.—Same tumor and series as in Fig. 3 except incubation time is 1 hour. The alkaline phosphatase activity is most intense in the peripheral cells of the islands. The stroma and vessels are entirely negative. × 120.

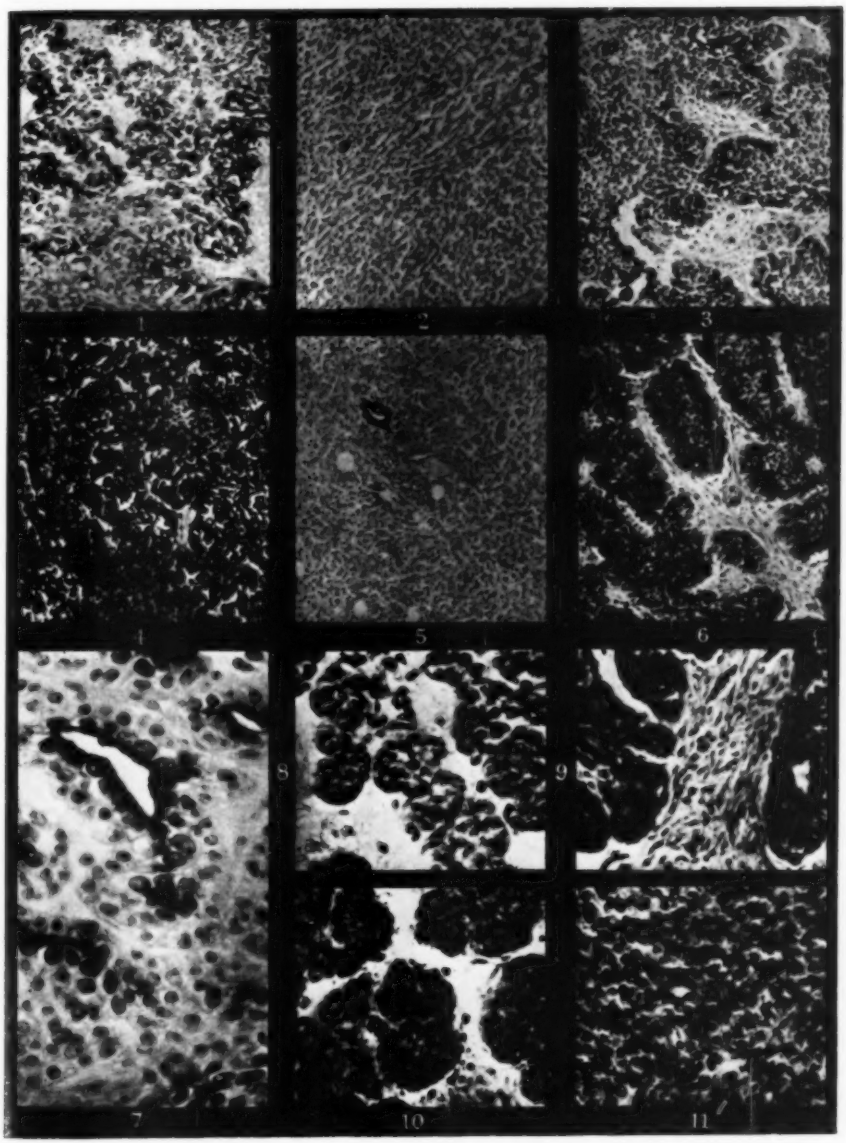
FIG. 7.—Tumor H2712 transplanted into C3H (Series II) under high power magnification. Note the peculiar and intense distribution at the borders of cells lining the lumens. In the center is also seen Gomori positive acellular material in a very small lumen. A small globule of positive material seen in a cell in the lower third, right. × 500.

FIG. 8.—A hematoxylin and eosin preparation of Tumor E0771 showing loose reticular stroma and islands of tumor tissue with small lumens. × 120.

FIG. 9.—A hemotoxylin and eosin preparation of Tumor H2712 showing in the central portion a dense stroma. Tumor islands are composed of closely packed cells and acinar structures. × 120.

FIG. 10.—A hemotoxylin and eosin preparation of Tumor dbrB showing the more solid and lobular structure with a loose cellular stroma with many large thin-walled vessels. The cells are smaller than in Fig. 8 and 9 and there is less tendency to lumen formation. Small lumens can be seen in the center of lobules. × 120.

FIG. 11.—A hemotoxylin and eosin preparation of Tumor 15091a showing a dense solid tumor. Cells irregular in size and shape. An occasional lumen is shown in the center. × 120.



Pearson and Richardson.

Histology and enzyme reaction in the tumor.

It would be highly desirable if one could relate certain histological and functional features to the enzymatic histochemistry of the tumor. However, in order to make such a comparison a large variety of tumors would have to be studied in inbred strains of mice and with comparable methods by several investigators. It is hoped that the following comparative descriptions might be of some value to future investigators in the clarification of the problems involved.

A visual comparison of the phosphatase activity of three tumors E0771, 15091a and dbrB is shown in Fig. 1 to 6. Fig. 1, 2, 3, show Tumors E0771, 15091a and dbrB respectively at 15 minutes and Fig. 4, 5, 6 at 60 minutes incubation period. It is quite evident that 15091a exhibits no alkaline phosphatase activity and further extension of the incubation time to $2\frac{1}{2}$ hours showed no reaction except in the vessels.

In Series I Tumor E0771 is composed of irregular islands of tumor tissue. These vary in size and shape. They are composed of large cells with large vesicular nuclei which also vary in size and shape. Numerous mitotic figures can be seen throughout. The islands are fairly solid with the exception of small areas where lumens can be seen. There seem to be attempts to form tubular structures. The larger lumens are lined by several rows and layers of cells. Most of the lumens are very small. The interstitial tissue is rather sparse and composed of loose reticular material with many large thin-walled vessels. Fig. 8 shows a photomicrograph of this tumor stained by hemotoxylin and eosin showing the islands of tumor tissue with an attempt at luminal and tubular formation. The interstitial tissue seen to the left is loose and reticular in nature.

The phosphatase reaction began during the first minute of incubation time with a blackening or graying of the cytoplasm. This gave a gray appearance to the slide except in such areas where the blackening is much more pronounced. At 5 minutes this process is more accentuated. The 15 minute incubation time is shown in Fig. 1 and represents a graying and intense irregular blackening in many areas of the slide. An attempt was made to see what cells or portion of the islands that would become intensified more quickly. It seemed that those acini in which there were several layers present revealed the most intense activity. Also areas of cells of those islands adjacent to vascular channels, showed the most intense activity. Very distinct and abrupt deposits of sulphide can be seen in cells undergoing early degeneration. Here the cell membranes are also exaggerated. With the exception of three instances, no very definite pattern of activity could be ascribed to this tumor. Not much could be gained by studying the tumor at 1 hour because much of the detail is obscured by the intense blackening. The interstitial tissue and vessels showed no activity.

Tumor H2712 in Series II was similar histologically to E0771. It differs somewhat in that the interstitial tissue is more dense and organized. It is composed of similar islands of tumor cells. The cells are large and have irregular and vesicular nuclei with numerous mitotic figures. There seems to be more attempt at lumen formation although these are small. There are no double rows of cells as shown in E0771. Large areas are fairly solid and show no evidence of lumen formation. A hemotoxylin and eosin section of the tumor is shown in Fig. 9. Small lumens can be seen in the tumor tissue. With the Gomori reaction as shown in the high power section in Fig. 7, it is seen that the luminal border of the acini give a very intense reaction at 15 minutes' incubation time. Similarly in the center

of the picture is seen an attempt at lumen formation in which the activity is high. The end point of the reaction occurs at 5 minutes. At this time enzyme activity can be demonstrated in many areas in the centres of the island. Large irregular clefts can be distinguished and stand out prominently by the activity of the enzyme of the cells lining the lumens. Intraluminal material which is not apparent in the hemotoxylin and eosin preparation shows intense activity. Small globules are present in the cytoplasm of many cells adjacent to lumens or attempted lumen formation. The connective tissue and vessels do not react.

Tumor dbrB in Series III under hemotoxylin and eosin stain shows a more lobular distribution than either E0771 or H2712 and is shown in Fig. 10. The cells and nuclei are smaller than those described for the other series. Numerous mitosis are seen. Lumen formation is sparse but where they are seen they are similar to the other 2 tumors. The interstitial tissue is composed of loose connective tissue, connective tissue and large thin-walled vessels.

With graded Gomori stains the reaction has an " end point " at 15 minutes. Here the areas of activity are irregular thoughout the tumor. Some activity is present in the small lumens but this is not marked. There is a tendency for increased activity at the periphery of the lobule as shown in Fig. 3 and 6. At 1 hour incubation time (Fig. 6) the reaction becomes much more intense. The loose supporting connective tissue and vessels show no activity.

In Series IV Tumor 15091a differs somewhat from the other 3 in that it is a fairly solid tumor not composed of islands or lobules. There are numerous mitosis and the cells vary more in size and shape. The nuclei are for the most part vesicular, very rarely is there evidence of lumen formation. Fig. 11 shows the hemotoxylin and eosin preparation with a small alveolar formation in the center.

With graded Gomori reactions as carried out on the other tumors, this shows a consistently negative reaction in all its incubation times up to 2½ hours. Fig. 2 shows the absence of phosphatase activity at 15 minutes and Fig. 5 at 1 hour. However, in this tumor the vessels show a consistent positive reaction as seen in Fig. 5. Also here is shown in the center, a few small lumens.

Other Organs..

Several other organs were studied in a similar manner to that of the tumor. The spleen revealed no change until 15 minutes of incubation and then the reaction began with the blackening of the central vessels of the follicle. At 1 hour this showed rather marked activity. The cells of the peripheral portion of the nodules showed most intense activity. There is no essential difference between the various series.

The liver shows, for the most part, two types of reaction. The commonest begins at the lining of the sinusoids involving the Kupfer cells. The other type of reaction begins at the periportal areas without involvement of the sinusoids. Although the reaction begins for the most part at the 15-minute incubation period it varies considerably as to extent and localization.

The kidney sections are similar in all series and begins in the brush border of the convoluted tubules. The ovary begins in most series with the 5 minute incubation time in the theca externa which shows progressive activity to 1 hour. Small stromal vessels also show activity. All the series were approximately the same. The reaction in the testes began at 5 minutes and became more intense at

1 hour incubation. For the most part it only involved the capsule surrounding the spermatic epithelium. Very little reaction was present in the adrenals and occurred for the most part at 1 hour incubation time where the reaction was present in the vessels in the glomerular zone.

DISCUSSION.

By means of graded Gomori reactions a more complete picture could be obtained concerning differences between 4 tumors arising in the breast tissue of mice. These tumors have been transplanted for many generations to their original hosts and F_1 hybrids with a 100 per cent take. Thus a certain homogeneity of the tumor strain should be expected. The three tumors E0771, H2712 and dbrB are fairly identical in histology, as each is an adenocarcinoma with attempts at acinar formations. This latter feature is slightly more developed in E0771 and H2712 than in the dbrB. One difference that occurs in the E0771 is the presence of double layers of tumor cells in the attempt at acinar formation. In the 15091a the tumor is solid with very few small acini.

The overall phosphatase reaction was very intense in E0771, very much less in H2712 and bdrB and none in 15091a. This was evident in spite of the fact that the rate of growth of the tumor was about the same in all the series. Numerous mitoses were present in all the tumours. Hard, Pratt-Thomas and Belkin (1948) felt that there was a loss of alkaline phosphatase activity in the more anaplastic tumor cells. Although it would seem in our series that the anaplastic Tumor 15091a having no reaction would point toward such contention, the E0771 is a very rapidly growing tumor with numerous mitoses. We would prefer to delay our judgment on this issue until a larger variety and strains of tumors have been studied.

An interesting and significant paper which has come to our attention as this work was completed was the observation of Shelton (1952). This investigator found a tumor in a 6-month-old Strain A female mouse that had been exposed to 400 r whole body X-radiation on the date of birth. Lymphoma 1 was propagated in A mice from a large tumor mass located in the anterior mediastinum. Lymphoma 2 was propagated by subcutaneous injection into Strain A mice of fragments of lymph nodes from a mouse bearing the original tumor. Strain 1 grew as a localized tumor with local invasion but no metastasis, but Strain 2 reacted as an acute leukemia with rapid growth infiltration of the organs and high peripheral count.

In determination of the alkaline phosphatase activity it was found the Lymphoma 1 showed no reaction while Lymphoma 2 showed a reaction as expressed by 5 mg. phosphorus per milligram of tissue per hour. No detectable reaction could be seen by the Gomori method in Lymphoma 1 whereas Lymphoma 2 was strongly positive. Here we have a rapidly growing persumably anaplastic tumor showing an increase in alkaline phosphatase reaction.

In our laboratory we have also determined the alkaline phosphatase activity as expressed in gamma of phosphorus per milligram of nitrogen at 30 minutes incubation time in a large series of these 4 mammary tumours. We have found that the specific activity of alkaline phosphatase thus expressed in gamma of phosphorus was 701 ± 30 in the E0771 tumor, $157 \pm 3{\cdot}4$ in the dbrB tumor, $30 \pm 4{\cdot}0$ in the H2712 and only $5 \pm 0{\cdot}6$ in the 15091a tumor. This corresponds to the histochemical findings of a very high and low values in E0771 and 15091a

respectively. There is a discrepancy in the H2712 tumour which shows an "end point" at 5 minutes in contrast to dbrB which shows it in 15 minutes. That this discrepancy is probably due to differences in the localization of enzyme and incubation times, thus there is a sharp localization of enzyme activity in the luminal border of the cells of H2712 which is not the case in dbrB. These tumours were transplanted into the same hosts and under the same conditions as the four series reported here. In the chemical reaction the incubation times were taken only at 30 minutes whereas we were concerned with several incubation times in our histochemical reactions.

The distinctive localization of Tumor H2712 is of interest in so far as its histological appearance is quite similar to all except 15091a. The fact that the luminal borders of the cells as well as accumulation into the lumen of Gomori positive material which is acellular suggests a secretory process which has been retained by this tumor.

CONCLUSIONS.

1. Four series of transplantable tumors arising from the breast in mice were transplanted to their original hosts and F_1 hybrids.

2. Enzyme activity as expressed by graded Gomori reactions with 4 incubation times indicated overall differences in alkaline phosphatase activity from a very intense reaction to no reaction.

3. Variation in localization of the reaction in one tumor (H2712) was striking as there was accumulation of activity in the luminal border and activity within lumens suggesting a secretory process.

4. Very little difference histologically could be seen in 3 tumors (E0771, H2712, and dbrB) to account for variations in overall intensity as well as such localization difference that existed.

5. The biochemical data seem to correlate very closely to the histochemical data, especially where wide differences exist.

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SURVEY OF PAPERS.

CURWEN, KENNAWAY and KENNAWAY have analysed the mortality figures for cancer of the lung and larynx in England and Wales over the period 1946–1949 by regions. They find that the mortality from cancer of the lung in both sexes, and of the larynx in males, increases with increasing urbanisation, but that cancer of the female larynx shows the reverse relationship. They emphasise the anatomical differences between laryngeal cancer in men and women, and discuss the significance of their findings in the light of the possible aetiology of cancer of the lung and larynx (p. 181).

KREYBERG finds that the histological types in a large series of lung tumours differ in their age curve and sexual incidence. He emphasises that lung cancer is a heterogeneous group histologically as well as biologically, a point of importance when considering aetiological factors (p. 199).

KREYBERG has analysed the Norwegian mortality statistics relating to lung cancer over the past years on the background of the different histological types occurring. He finds the ratio of the different types has remained about the same in women, the increase in lung cancer in women representing increased diagnostic efficiency. In males there has been a relative increase in the number of squamous cell, large- and small-cell carcinoma recently with the development of a pronounced deviation in mortality between the sexes (p. 209).

STEINER has studied curves made of the ratios of necropsies for malignant disease to all necropsies by age. He finds that the cancer to necropsy ratios for tumours of different sites within each organ system show similar curves, differing only in amplitude. The curves for each system are characteristic and differ from those for other systems (p. 215).

WARWICK and PHILLIPS find no difference in the incidence of cancer in Canadian Indians and the White population below 30 years of age; over this age the incidence is apparently less among Indians. They find that cancer of the skin is rarer in Indians, whereas carcinoma of the cervix is commoner and occurs earlier (p. 223).

BOYD and DOLL have compared the purgative histories of a series of gastro-intestinal cancer patients with a series of patients with diseases other than gastro-intestinal. They find no difference in the overall use of purgatives, but an appreciable excess amongst gastro-intestinal cancer patients only in the use of liquid paraffin (p. 231).

KEYSER has found that in a series of patients with various tumours treated with nitrogen mustard or irradiated, a favourable clinical response was generally associated with a fall in the blood serum polysaccharide towards normal levels, the level rising again with subsequent relapse in the clinical condition (p. 238).

MARSHALL and PEPLER describe a case of multiple mollusca pseudocarcinomatosa in which the lesions were unilateral. They discuss the nature of these tumours and suggest that the condition may be of naevoid origin (p. 251).

BLACKBURN finds nothing to suggest that either the prognosis or the clinical course of acute leukaemia is altered by adding stilboestrol to other therapy (p. 255).

AMBROSE presents a consideration of the problems of tumour formation and growth in terms of certain simple mathematical relationships (p. 259).

HIEGER and ORR give an account of the induction of sarcoma in mice on injection of commercial cholesterol dissolved in olive oil. A high degree of purification did not affect the carcinogenicity of the cholesterol. Stock mice were found to be about twice as sensitive as C57 mice to carcinogenesis by commercial cholesterol (p. 274).

GHADIALLY and GREEN find that cortisone applied locally from the commencement of dimethyl benzanthracene painting almost completely inhibits papilloma formation in mouse skin. The same effect is produced if the cortisone is allowed to act during the "developmental" phase of carcinogenesis, but it is quite ineffective when applied solely during the "pre-induction" and "induction" phases (p. 291).

COMMINS, COOPER and LINDSEY have extracted the condensable products of cigarette smoke and examined them by chromatography followed by absorption spectrophotometry. They find absorption peaks characteristic of anthracene and pyrene in the extract. They also find the temperatures attained in the burning end of a cigarette to fluctuate between 650 and 700°C, temperatures sufficiently high to cause pyrolysis of simpler compounds of polycyclic hydrocarbons (p. 296).

COHEN and COHEN have determined the effective doses of irradiation for C3H adenocarcinoma growing in three apparently uniformly susceptible groups of mice. The show that the radiosensitivity of a tumour is a quantitative measure of subliminal host-resistance resulting from immunogenetic differences in the host-tumour relationship, including extrachromosomal factors (p. 303).

COHEN and COHEN find that homoplasts of C3H mammary adenocarcinoma attenuated by irradiation prior to implantation in C3H mice show increased radiosensitivity when subsequently treated *in situ*. When the previously attenuated homoplasts are transplanted from their original hosts to new hosts the curative dose reverts to that of the unattenuated tumour (p. 313).

GOLDFEDER has investigated the radiosensitivity and ability to induce a resistant state in mice of the same strain of two transplantable adenocarcinomas. The results emphasise the part played by the genetic relationship between the tumour and host in the induction of resistance to the appearance of tumours. Under the experimental conditions described it was not possible to induce immunity in homozygous hosts to endogenous tumours by irradiation of these tumours (p. 320).

JACKSON finds that the Walker carcinosarcoma, at first completely inhibited by the administration of triethylene melamine to rats bearing implants, subsequently becomes resistant to the drug and growth of the tumour recommences. The resistant tumour shows no tendency to recover its sensitivity (p. 336).

WOODHOUSE has examined the epidermal proteins from mice, rats, rabbits and guinea-pigs which had been painted with 3 : 4-benzpyrene during life. He finds similar amounts of bound hydrocarbon in all the animal species, though they vary considerably in their response to this hydrocarbon as a carcinogenic agent. He has also tested other hydrocarbons, less carcinogenic to mice, and again, comparable amounts were found to be bound to the epidermal proteins (p. 346).

HAREL and OBERLING present new data on certain ultramicroscopic granules and rods originally noted in leukoblasts of human leukemia and to which the term ultrachondrioma has been applied. They have noted the presence of these particles in normal as well as malignant cells, and discuss their significance (p. 353).

NEISH finds that sera from rats bearing transplanted tumours are less effective than normal rat sera for reducing 2,3,5-triphenyltetrazolium chloride (TTC) as determined polarographically, and that there is a highly significant negative linear correlation between percentage TTC reduced and tumour weight (p. 361).

LAJTHA, OLIVER and ELLIS have studied and timed the period of DNA sythesis in the intermitotic cycle of human bone marrow cells *in vitro*, using the incorporation of ^{32}P and adenine

^{14}C as indicators. They find no difference in the times for the same types of cells, whether in normal marrows or marrows from patients with leukaemia, but blast cells from acute leukaemias show a markedly slower rate of DNA synthesis than promyelocytes and myelocytes. They also find that 5000 r X-ray irradiation inhibits DNA synthesis immediately and completely (p. 367).

MOODIE and REID have studied the phosphorescence emission spectra of a series of carcinogens with the same basic ring structure and find that carcinogenic power correlates with the position of the lowest triplet level. They also find that spectral shifts in complexes between naphthacene and carcinogens correlate well with carcinogenicity (p. 380).

PEARSON and RICHARDSON have found marked differences in the alkaline phosphatase activity, as shown by Gomori reactions, in four transplantable mammary tumours in mice (p. 384).